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Research Article

Caffeine Pretreatment Increases Survival of DBAXC57BL Mice Exposed to Different Doses of γ – Radiation by Glutathione Elevation

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Abstract

Humans are exposed to ionizing radiations from various sources including background, air or space travel and diagnostic and cancer therapy. The deleterious changes induced by ionizing radiations can be reduced using different pharmacophores. The effect of 80 mg/kg body weight of caffeine was studied on the radiation-induced sickness and mortality in DBAxC57BL mice exposed to 7 to 13 Gy of γ -irradiation. Treatment of mice with caffeine one hour before irradiation delayed the onset of mortality and reduced the symptoms of radiation sickness when compared to saline treated irradiated controls. Caffeine provided protection against both the gastrointestinal and hemopoietic deaths. However, animals of both the Caffeine and Saline pretreated irradiation groups did not survive up to 30 days post-irradiation beyond 11 Gy irradiation. The LD50/30 was found to be 9.4 Gy for the Saline and 10.2 Gy for caffeine pretreated irradiation group, respectively with a dose reduction factor of 1.1. The ability of caffeine to protect mice against the radiation induced mortality is due to increase in glutathione accompanied by a reduced lipid peroxidation on 31days in the survivors. Caffeine protected the DBAxC57BL mice against radiation induced sickness and mortality by increasing glutathione and depleting lipid peroxidation.

Keywords: Caffeine; survival; dose reduction factor; glutathione; lipid peroxidation; radiation sickness

Introduction

Radiation is an important modality in the treatment of cancer and in some instances, it may be the single best agent for treatment. However, a major problem associated with the cancer radiotherapy is the severe side effects resulting from the normal tissue damage and it is known to induce second malignancies in the survivors [1]. This indicates the need to protect normal tissues against the radiation-induced damage. The use of radioprotectors will also be able to increase the patient's tolerance to radiotherapy and ameliorate the symptoms of radiation sickness. Historically, the sulphydryl compounds were among the first radioprotectors to be identified, where cysteine a natural amino acid was reported to protect mice against the radiation-induced sickness and mortality [2]. Since then, several compounds with varied chemical structures and pharmacological properties have been screened for their radioprotective ability in mammals. However, these compounds appear to produce serious side effects and are toxic at the doses required for radioprotection [3,4]. In addition to its utility in the cancer treatment, an efficient, non-toxic radioprotector could also prove useful in occupational settings, where ionizing radiations are in frequent use (e.g., defense, airline, military and research personnel, nurses, dental assistants, radiotherapy and nuclear medicine technicians, etc.) or in accidental exposures which leave radioactivity in the environment (viz. Three Mile Island, Chernobyl, Goiania, and Fukushima) and also during space travel to protect astronauts from the effects of high doses of radiation associated with solar flares [4].

Recently, there has been a tremendous increase in the terrorist activities worldwide. Despite tight regulations regarding the nuclear fissile material, it is feared that some of the terrorist organizations may have access to nuclear fissile materials. To spread terror among

the innocent public as well as the administration the committed terrorists may use nuclear fissile materials without hesitation causing untold misery to the human beings. This indicates urgent need to devise countermeasures to protect the public from the deleterious effect of ionizing radiation by screening non-toxic radioprotectors, which will also be useful in the cancer patients undergoing radiation treatment [4, 5].

Dietary antioxidant compounds have recently been the focus of attention, as they have been found to be of immense use in preventing and ameliorating various human ailments and diseases. Further, human beings have been consuming these compounds since time immemorial and the major advantage of these dietary ingredients over the synthetic drugs lies in the fact that most of them have a low effective dose to high toxic dose ratio. This property gives immense advantage as it can be easily recommended for human trials and at lesser costs when compared with their synthetic counterparts [4, 6].

Caffeine, a nervous system stimulant belongs to methylxanthine class of psychoactive drugs. It is a major constituent of coffee, and other beverages including tea which also contains some amount of caffeine. Caffeine has been reported to be a potent antioxidant and a free radical scavenger. It has also been found to protect the biological molecules against the free radical-induced damage, chemical and radiation-induced tumorigenesis in mice. Caffeine has been used as an adjuvant analgesic in combination with acetaminophen, aspirin and ibuprofen in clinics. The caffeine has been shown to exert a wide variety of effects on DNA damage induced by UV and ionizing radiations, depending upon pre- or post-irradiation administration and its concentration. It has also been reported to potentiate UV-induced DNA damage, when administered after irradiation, while its presence before or during irradiation elicited protection in a wide range of test systems like bacteria, yeast, cultured cells, plant seeds and mouse [7-15].

The lessons from the experience with radioprotectors worldwide are that the animal studies with death as the end point is the most confirmatory. The survival after 30 days of lethal whole- body irradiation distinctly indicates the capacity of pharmacophores to be tested for their ability to modulate the recovery and regeneration of the gastrointestinal epithelium and the hemopoietic progenitor cells in the bone marrow, the two most radiosensitive organs that are essential for sustenance of life [4]. Studies carried out by George et al. [7] have shown that caffeine when administered before irradiation protected the animals against the radiation-induced mortality and sickness. However, the dose modification factor (DMF) has not been reported. The DMF is an important aspect in the radiobiology as it clearly gives indication of the drug's quantitative and qualitative capacity in enhancement of tolerance of tissues to radiation and its effect on amelioration of the radiation-induced sickness and mortality [4]. Therefore, the present study was carried out to obtain an insight into the effects of caffeine on the survival and modulation of certain biochemical parameter in the DBAxC57BL mice exposed to different doses of γ-radiation.

Materials and Methods

The handling and care of animals were done according to the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India) guidelines. Eight to ten weeks old DBAxC57BL mice of either sex (1:1 ratio) weighing 20 to 23 g were selected from an inbred colony. The animals were kept at a temperature of 23 ± 2°C, humidity (50 ± 5 %) and 10 and 14 h of light and dark, respectively. The animals were fed with sterile mice food and had free access to water. Generally, four animals were put in a sterilized polypropylene cage containing sterile paddy husk (procured locally) as bedding during the experiments. The animal ethical committee of Manipal University, Manipal, India approved the study.

Preparation of the Drug

The caffeine or 1,3,7-Trimethylpurine-2,6-dione procured from Sigma Chemical Co. (St. Louis, USA) was dissolved in sterile distilled water immediately before use.

Mode of administration

The animals were administered with 0.01 ml/g b. wt. of sterile physiological saline or caffeine intraperitoneally.

Experimental

The animals were divided into the following groups:

Saline+irradiation group

The animals of this group were administered with sterile physiological saline before irradiation.

Caffeine+irradiation group

The animals of this group received a single dose of 80 mg/kg b. wt. Caffeine before irradiation [7].

Irradiation

One hour after the administration of saline or caffeine, the prostrate animals were placed in the specially designed well ventilated acrylic restrainers and immobilized by inserting cotton plugs. The restrainer was placed on the irradiation table and animals were whole body exposed to 0, 7, 8, 9, 10, 11, 12 and 13 Gy of ^{60}Co γ-radiation (Theratron, Atomic Energy Agency, Canada). A batch of six animals was irradiated each time at a dose rate of 1.66 Gy/min at a source to animal distance (midpoint) of 70 cm. Immediately after the irradiation, the animals were sorted into individual polypropylene cages. The animals of both Saline+irradiation and Caffeine+irradiation groups were daily monitored for the development of symptoms of radiation sickness, and mortality if any. A total of 9 male and 9 female animals were used for each dose of radiation for each group and 324 animals of both sexes in equal

ratio were utilized to complete the whole experiment. The dose reduction factor (DRF) was calculated by the method of Miller and Tainter [16].

DRF =LD50/30 of the Caffeine+irradiation group/LD50/30 of the Saline+irradiation group

BIOCHEMICAL ESTIMATION

The animals from both the groups, which survived up to 30 days were killed by cervical dislocation on the 31st day, after exposure and were perfused with ice cold saline transcardially. The whole liver from each surviving animal was removed, blot dried, weighed and a 10% homogenate was prepared in ice-cold 0.2M sodium phosphate buffer pH 8.0 using a homogenizer (Yamato LSG LH-21, Japan).

Protein estimation

Total proteins were estimated by Lowry et al. [17] method using bovine serum albumin as the standard.

Glutathione

Glutathione (GSH) contents were measured by the method of Moron et al. [18]. Briefly, proteins were precipitated by 25% TCA, centrifuged and the supernatant was collected. The supernatant was mixed with 0.2 M sodium phosphate buffer pH 8.0 and 0.06 mM 5, 5-dithio2-nitrobenzoic acid and incubated for 10 minutes at room temperature. The absorbance of the sample/s was read against the blank at 412 nm in a UV-Visible Spectrophotometer (Shimadzu UV-260, Shimadzu Corp, and Japan) and the GSH concentration was calculated from the standard curve.

Lipid Peroxidation (LOO)

LOO was measured by the method of Buege. et al. [19]. Briefly, the tissue homogenate was mixed with TCA-TBA-HCl. The mixture was heated for 15 min in a boiling water bath and centrifuged. The absorbance was recorded at 535 nm using a UV-Visible Spectrophotometer (Shimadzu UV-260, Shimadzu Corp, and Japan). The lipid peroxidation has been expressed as MDA in nM per mg protein.

Analysis of data

The statistical significance between the treatments was determined using the "Z" test for the survival studies and the student's t-test was applied for glutathione and lipid peroxidation. The Microsoft excel and Origin 8.5 (OriginLab Corporation, Northampton, MA, USA) statistical softwares were used for data analyses.

Results

The results are expressed as mean ± SEM (standard error of the mean) and presented as (Figures 1-5) and (Table 1).

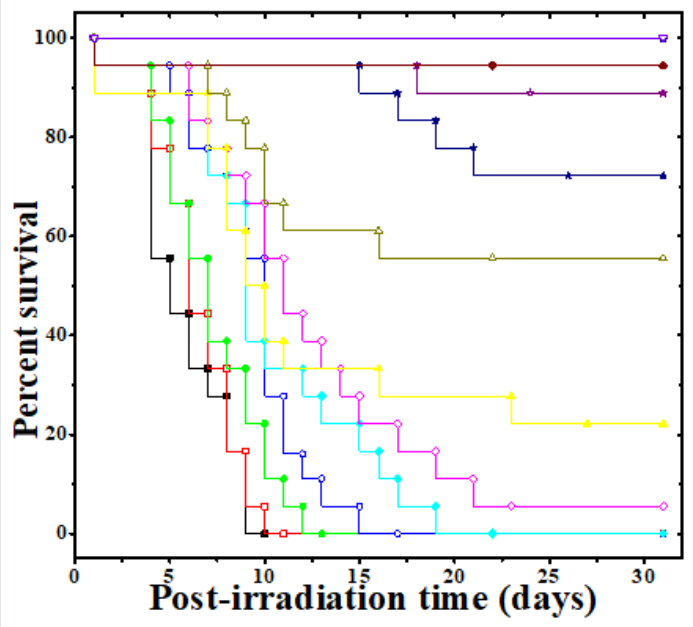

Figure 1: Kaplan Meier's estimate of survival of mice treated with 80 mg/kg b. wt. of caffeine before exposure to different doses of whole body γ -radiation. Closed down triangles: Saline+sham-irradiation; Open down triangles: Caffeine+sham-irradiation; Closed pentagons: Saline+irradiation (7 Gy); Open pentagons: Caffeine+irradiation (7 Gy); Closed hexagons: Saline+irradiation (8 Gy); Open hexagons: Caffeine+irradiation (8 Gy); Closed stars: Saline+irradiation (9 Gy); Open stars: Caffeine+irradiation (9 Gy); Closed uptriangles: Saline+irradiation (10Gy); Open uptriangles: Caffeine+irradiation (10Gy); Closed diamonds: Saline+irradiation (11Gy); Open diamonds: Caffeine+irradiation (11Gy); Closed circles: Saline+irradiation (12 Gy); Open circles: Caffeine+irradiation (12Gy); Closed squares: Saline+irradiation (13Gy) and Open squares: Caffeine+irradiation (12 Gy).

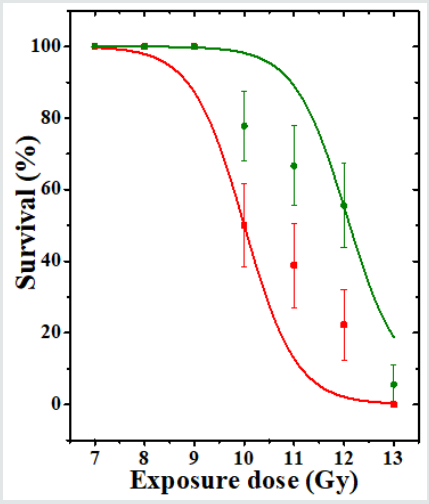

Figure 2: Effect of caffeine treatment on the gastrointestinal deaths (10 day) in mice exposed to different doses of γ-radiation. Squares: Saline+irradiation and Circles: Caffeine+irradiation.

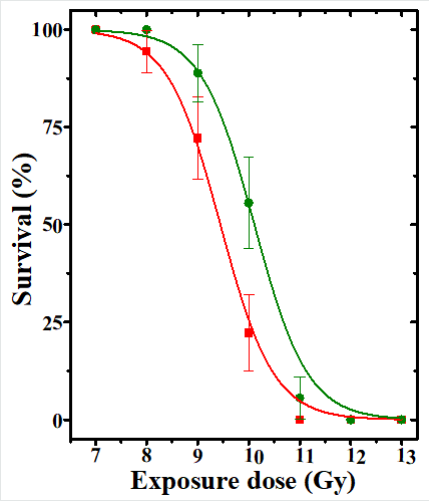

Figure 3: Effect of caffeine treatment on the hematopoietic deaths (30 day) in mice exposed to different doses of γ-radiation. Squares: Saline+irradiation and Circles: Caffeine+irradiation.

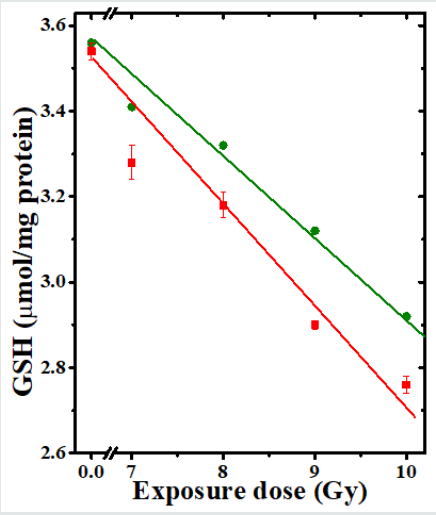

Figure 4: Effect of caffeine treatment on the glutathione contents in the liver of mice exposed to different doses of γ-radiation. Squares: Saline+irradiation and Circles: Caffeine+irradiation.

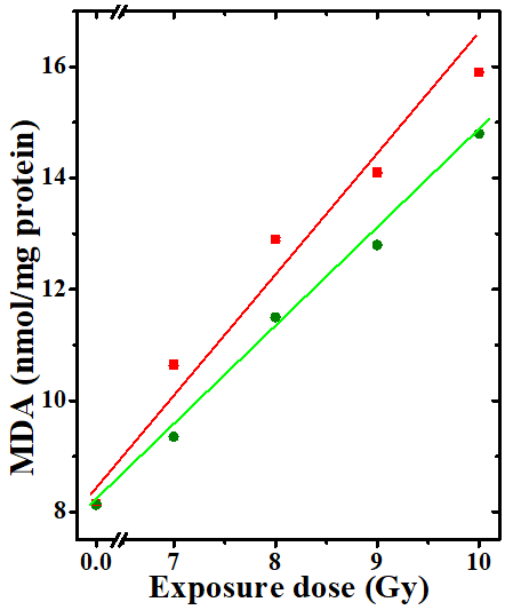

Figure 5: Effect of caffeine treatment on the lipid peroxidation in the liver of mice exposed to different doses of γ-radiation. Squares: Saline+irradiation and Circles: Caffeine+irradiation.

Table 1: Modulation of the radiation-induced changes in glutathione and lipid peroxidation in the liver of DBAxC57BL mice exposed to different doses of γ -radiation after 30days of irradiation. a: $p < 0.01$, b: $p < 0.001$.

Exposure dose	Glutathione (μmol/mg protein)		Lipid peroxidation (nmol/mg protein)	
	Saline + irradiation	Caffeine + irradiation	Saline + irradiation	Caffeine + irradiation
0	3.54±0.02	3.56±0.04	8.15±0.02	8.13±0.03
7	3.28±0.04	3.41±0.03a	10.65±0.02	9.35±0.01b
8	3.18±0.03	3.32±0.02b	12.90±0.03	11.5±0.04b
9	2.90±0.01	3.12±0.02b	14.10±0.01	12.8±0.02b
10	2.76±0.02	2.92±0.02b	15.90±0.01	14.8±0.02b

Survival studies

The animals of Saline + irradiation group exhibited signs of radiation sickness within 2-4 days after exposure to different doses of γ -radiation depending on the irradiation dose. The main symptoms included reduction in the food and water intake, irritability, epilation, weight loss, emaciation, lethargy, diarrhea, and ruffling of hairs. A few animals also exhibited facial edema between one and two weeks after exposure to doses above 10 Gy. Some of the animals exhibited paralysis and difficulty in locomotion during the second week after exposure to doses above 9 Gy. The severity of the symptoms increased and advanced with the increase in radiation dose.

The results are expressed as percent survival after exposure to various doses of γ-radiation. The whole-body irradiation of mice to 7Gy did not induce mortality in both the groups (Figure 1). However, with the further increase in exposure dose, the survival declined in a dose dependent manner and a nadir was reached after 11Gy exposure and no survivors were recorded beyond 19 days post-irradiation after exposure to 12 and 13Gy. The increase in the exposure dose also resulted in an advancement in the onset of mortality (Figure 1). The survival was plotted and the data for day 10 and 30 mortality were fitted on a sigmoid curve (Figure 3 and 4). The LD50/30 was found to be 9.4 Gy for the Saline + irradiation group (Figure 4).

The treatment of mice with 80 mg/kg caffeine before one hour of irradiation delayed or reduced the severity of radiation-sickness symptoms and decreased the radiation-induced mortality when compared with the concurrent Saline +irradiation group. Caffeine pre-treatment protected mice against both the gastrointestinal (GI) and hemopoetic deaths as evidenced by the greater number of survivors at the end of 10 and 30days post-irradiation when compared with the concurrent Saline +irradiation group. The caffeine pre-treatment increased the animal survival by 5.55 % after exposure to 11 Gy, while no survivors could be observed by 30days post-irradiation in the Saline +irradiation group (Figure 4).

Similarly, treatment of mice with caffeine before exposure to 8, 9 and 10 Gy reduced the 30day mortality by 1.06, 1.23 and 2.5, fold when compared with Saline +irradiation group (Figure 4).

The results were statistically significant for 10 ($p<0.02$) and 11Gy ($p<0.0001$) exposure when compared with the concurrent Saline +irradiation group. The LD50 /30 was found to be 10.2 Gy, resulting in an increase of 0.8 Gy when compared with the Saline +irradiation group. The dose reduction factor (DRF) was found to be 1.1.

Glutathione

The results are expressed as glutathione (GSH) contents µmol/mg protein (Table 1). GSH contents remained unaltered in the Saline +sham-irradiation group (0Gy). Similarly, the administration of caffeine alone before sham-irradiation did not alter the glutathione contents. The exposure of animals to different doses of radiation resulted in a significant and dose dependent decline in the GSH contents in the Saline+ irradiation group (Figure 4). However, caffeine pretreatment elevated the GSH contents significantly when compared to the concurrent Saline + irradiation group. This increase was 1.04, 1.05, 1.1 and 1.1 folds higher than that of 7, 8, 9 and 10 Gy concurrent Saline+irradiation group, respectively. The GSH contents were below normal in the Saline + irradiation and Caffeine+irradiation groups (Figure 4).

Lipid Peroxidation

The lipid peroxidation is expressed in terms of nmol MDA/mg protein (Table 1). LOO remained unaltered in Saline+ sham-irradiation group. The administration of caffeine alone before sham-irradiation (0Gy) did not increase the MDA concentration and was almost akin to the Saline + sham-irradiation group (Table 1). The induction of LOO increased with the increase in irradiation dose in both the Saline + irradiation and Caffeine + irradiation groups and a peak level was observed at 10 Gy irradiation (Figure 5). The caffeine pretreatment significantly reduced the LOO induction in the Caffeine+ irradiation group thereby protecting against the radiation-induced lipid peroxidation at all the exposure doses studied and it was 1.14, 1.12, 1.1 and 1.1 folds lower for 7, 8, 9 and 10 Gy Saline + irradiation group, respectively when compared with the concurrent Saline + irradiation group (Table 1). Inspite of decline in the LOO by caffeine, the LOO values were higher than the Saline + sham-irradiation group (Figure 5).

Discussion

There is a continued interest and a need is felt for the identification and development of non-toxic and effective radioprotective compounds, which could protect humans against the genetic damage, mutation, alterations in the immune system and teratogenic effects of ionizing radiations. An efficient, non-toxic radioprotector may prove as a countermeasure in nuclear accidents, and intentional terror attacks [4, 5]. The good radioprotector are also needed to protect the occupational workers and patients exposed to ionizing radiations during diagnostic and therapy. The radioprotectors would be useful during whole body X-ray screening of frequent travelers at airports, which adds extra burden to their radiation exposure. This indicates the need to study the radioprotective effect of a pharmacophore in different study systems. Therefore, the radioprotective ability of caffeine was evaluated in the DBAxC57BL mice exposed to different doses of γ-radiation.

A single whole-body exposure of mammals to ionizing radiation results in a complex set of symptoms whose onset, nature, and severity are a function of both total radiation dose and radiation quality. It is a well-known fact that ionizing radiations deposit energy in the cell randomly within 10^{-18s} [20]. At the cellular level, ionizing radiations induce damage in the biologically important macromolecules such as the DNA, RNA, proteins, lipids and carbohydrates of the various organs [20, 21]. This damage in the cellular milieu is triggered by the formation of free radicals by ionizing radiations [22]. The exposure of DBAxC57BL mice to different doses of γ-radiation resulted in the triggering of symptoms of radiation sickness and mortality depending on the irradiation dose [23]. A similar observation has been made in DBAxC57BL mice treated with mangiferin earlier [5]. While some damage may be expressed early, the other may be expressed over a period depending upon the cell kinetics and the radiation tolerance of the tissues. The proliferating cells are highly sensitive to the effect of ionizing radiation; therefore, the effect of whole-body irradiation is mainly felt by the highly proliferating germinal epithelium, gastrointestinal epithelium and the bone marrow progenitor cells. The germinal epithelium does not contribute to life supporting functions of the exposed individual and therefore does not contribute to the survival, whereas the gastrointestinal epithelium and the bone marrow progenitor cells are crucial for the sustenance of life and any damage to these cells will impair the normal physiological processes drastically causing adverse impact on the survival [4, 5, 24, 25]. The gastrointestinal epithelium is less sensitive than the bone marrow progenitor cells but as the cell transit time is quick, it is expressed earlier than the hemopoietic syndrome. In mice death within 10 days post-irradiation is due to the gastrointestinal damage [4, 26-32]. The bone marrow stem cells are more sensitive to radiation damage than the intestinal crypt and the hemopoietic syndrome occurs at lower doses and is manifested as hemopoietic stem cell depletion, followed by the depletion of mature hemopoietic and immune cells [4, 5, 24, 25]. However, the peripheral blood cells have a longer transit time than the intestinal cells and hence the gastrointestinal syndrome appears earlier than the bone marrow syndrome and in mice, death due to irradiation from 11 to 30 days post-irradiation is due to the hemopoietic damage inflicted by radiation [4, 5, 23, 26-32].

The pattern of survival in caffeine group was akin to that of Saline + irradiation group except that the mortality was reduced. This clearly indicates the effectiveness of caffeine in arresting GI death, where the number of survivors for all the treatment

groups was higher than that of the Saline + irradiation group. The administration of 80 mg/kg caffeine resulted in the protection of mice and this reduction in GI death may also be due to the protection of intestinal epithelium, which would have allowed proper absorption of the nutrients. Caffeine has been reported to protect the mouse intestinal cells from radiation injury [33]. It has also been reported to ameliorate the detrimental effects of combined treatment of radiation and indomethacin on GI injury in mice [34]. Likewise, mangiferin has been reported to protect DBAxC57BL mice against the γ-ray induced radiation sickness and 10 and 30day mortality [23].

The treatment of mice with caffeine significantly reduced the bone marrow deaths in the Caffeine +irradiation group. This increase in 30day survival may be owing to the protection afforded by caffeine to the stem cell compartment of the bone marrow, which continued to supply the requisite number of cells in the survivors. Caffeine has been reported to protect mice against whole body lethal dose of irradiation [11]. The administration of caffeine has been reported to reduce the radiation-induced chromosomal aberrations and inhibit the radiation-induced single-strand breaks in the pBR322 plasmid DNA in a dose-dependent manner [8, 14]. The other radioprotective agents have been reported to protect mice against the GI and bone marrow deaths after exposure to different doses of γ-radiation [4, 5, 26-32].

The importance of the cellular membrane as a critical target in the enhancement of radiation-induced cell lethality has been emphasized [35]. Lipid peroxidation induced by radiation is known to be due to the attack of free radicals on the fatty acid component of membrane lipids [36]. Lipid peroxidation is considered to be an important effect of ionizing radiation on biological membranes [37]. While DNA damage causes the radiation-induced reproductive cell death, membrane lipids are thought to be critical targets in the interphase cell death [38]. Radiation-induced lipid peroxidation causes damage to the cellular membrane by altering the fluidity of the biological membranes which progressively leads to cell degradation and thereby affecting the biological defence mechanism [39]. It is reported that the product of lipid peroxidation, such as malonaldehyde (MDA), damages the enzyme system and DNA [40]. Lipid peroxidation has been used as an endpoint to study the action of oxidizing and free radical producing agents as well as to investigate the effects of intracellular radical scavengers. The caffeine administration significantly decreased radiation induced lipid peroxidation in the liver of survivors. The caffeine has been reported to inhibit the hydroxyl radical, peroxyl radical and singlet oxygen-induced membrane damage and the lipid peroxidation [9, 10, 12].

Several investigators have reported that lipid peroxidation start as soon as the supply of endogenous GSH is exhausted, and that the addition of the GSH promptly stops further peroxidation [41]. GSH is involved in numerous cellular reductive reactions [42]. It is related to the repair of radiation-induced free radicals by hydrogen atom donation, rejoining of DNA strand breaks by participating in enzymatic reactions as a cofactor, and in the repair of DNA damage, resulting in protection [42, 43]. The caffeine administration before irradiation resulted in a significant rise in the GSH level at all exposure doses in the liver of survivors when compared with the concurrent Saline +irradiation group. This elevation in GSH may be responsible for the decline in LOO and against the radiation-induced mortality. A similar effect has been observed earlier [10, 12].

The mechanism of radioprotective action of caffeine may be due to its antioxidant properties. Caffeine has been reported to be a scavenger of the hydroxyl radicals and singlet oxygen thereby resulting in the reduction in the radiation-induced damage to the cellular DNA [12]. In oxic conditions, caffeine readily accepts electrons with a rate constant of 1.5×10^{10} M^{-1S-1} whereas oxygen accepts one electron and forms superoxide with a rate constant 1.9×10^{10} M^{-1S-1}. This always results in the competition between oxygen and caffeine for availability of electrons. Caffeine molecules compete with oxygen for the radiation induced electrons and the removal of electron by caffeine could prevent the possible damage [7]. Caffeine has also been reported to possess activity similar to that of glutathione and significantly higher than that of ascorbic acid. Caffeine has been reported to be a better scavenger of OH radicals than both glutathione and ascorbic acid [9]. All these actions may be responsible for the increase in survival in the present study. In addition to that caffeine may have employed molecular pathways for its radioprotective activity. The whole-body exposure of mice to ionizing radiations has been found to trigger the activation of NF-κB, COX-2, TNF-α and MAPK [44-46]. The radioprotective action of caffeine seems to be mediated by inhibition of these cytokines as it has been reported to suppress the NF-κB, COX-2, TNF-α and MAPK activation earlier [47,48]. The whole-body exposure of mice has been reported to attenuate the Nrf2 expression [45]. Increase in the GSH contents by caffeine seems to be due to the upregulation of Nrf2 that subsequently protected mice against the radiation-induced sickness and mortality.

Since caffeine is consumed daily by human beings, its use and acceptability will not pose any problem in clinics and it may not produce untoward toxic side effects in patients. In fact caffeine administration has been reported to decrease the severe late toxicity of radiation in the cervical and endometrial cancer patients [49]. Similarly, caffeine administration has also been reported to ameliorate the radiation-induced skin reactions in mice without conferring protection to the tumor [50].

Conclusion

Caffeine has provided protection against the radiation induced sickness and mortality in the mice. The radioprotective action of caffeine seems to be due to increased GSH level and reduced lipid

peroxidation. The caffeine also protected the DBAxC57BL mice by inhibiting the radiation induced upregulation of NF-κB, COX-2, TNF-α and MAPK and depletion in Nrf2.

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Conflict of interest statement

Authors have no conflict of interest statement to declare.

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