# The radioprotective effects of carboxyfullerene $C_3$ on AHH-1 cell

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## ABSTRACT

**Purpose** To investigate the radioprotective effects of carboxyfullerene $C_3$ on AHH-1 cell and it's prospective as a novel radioprotectant.

**Materials and Methods** Carboxyfullerene $C_3$ was prepared by chemical synthesis and trypan blue rejection test was performed to detect its cytotoxicity to AHH-1 cell. Then different concentration of $C_3$ was used to treat AHH-1 cells after radiated with $^{60}$Co γ ray. Annexin-V/PI staining and flow cytometry assay were applied to assess the cell proliferation and apoptosis after irradiation.

**Results** $C_3$ showed little toxicity to AHH-1 cells with little change of trypan blue rejection rate during the drug concentration range 0-400 mg/L (*P>0.05*). We found in this study $C_3$ had good radioprotective effects to AHH-1 cell radiated with 1-8 Gy γ-ray. When the concentration was 10 mg/L, $C_3$ showed protection effects to AHH-1 cell irradiated with 4Gy γ-ray, which was enhanced with increase of $C_3$ concentration. When the final concentration reached 200-400mg/L, the cell survival rate after irradiation was similar to that of non-irradiated control cells(*P>0.05*).And the irradiation induced apoptosis and death rate were significantly lower than that of single radiation group cells(*P <0.01*). Moreover, we found the radio-protective effects of $C_3$ were time-dependant, and the best protection effects were observed when the $C_3$ was administered before irradiation (0-24 h).

**Conclusion** Carboxyfullerene $C_3$ has good radioprotective effects to AHH-1 cell,

which is dose-dependent， and the higher concentration of $C_3$ is, the better protective effects it shows. In the effective drug concentration range of this study, $C_3$ do little harm on the survival rate of AHH-1 cell, which suggest that $C_3$ as a novel promising radioprotectant deserve to be further investigated.

**[Keywords]:** Carboxyfullerene $C_3$; Radioprotectant; γ ray; AHH-1 cell

## Introduction

Finding the effective radioprotectant with low toxicity has always been one of hard and emphasized work in the field of medical science and radiation protection. There are several ways to exploit radioprotectants, among which free radical scavenger and antioxidants are two critical approaches [1-2].

Buckminsterfullerenes ($C_{60}$) have unique cage structure which allows them to interact effectively with free radicals and are characterized as effective free radical scavenger and antioxidants [3]. However, the poor solubility of native $C_{60}$ limits its application in biological research and medical therapy. Recently, several strategies have been developed to convert hydrophobic fullerene into water-soluble compounds by adding different groups to the fullerene's carbon cage. Carboxyfullerene ($C_3$) is a water-soluble trimalonic acid derivative of fullerene [4], and considerable research has been conducted recently and comprehensive information on the biomedical effects has been accumulated. It was reported that $C_3$ is an excellent free radical scavenger and an effective antioxidant both in vitro and in vivo, which confers some interesting properties to $C_3$ such as protecting neuronal cells from deterioration induced by excitotoxic injury [5,6], blocking the apoptosis induced by transforming growth factor-β in human hepatoma cells [7], and preventing the iron-induced oxidative injury in rat brain [8]. However, the effect of $C_3$ on protection against radiation is not clearly understood.

In this study, we firstly synthesized $C_3$ according to the method of document, then

biological tests were set out to explore the radioprotective effects of $C_3$ on AHH-1 human lymphoblastoid cells against γ ray radiation. In the meantime, we investigated the cytotoxicity, effective dose and the administer timing of $C_3$ to be used as a radioprotector. Our findings would provide the experimental basis for $C_3$ to be utilized a novel radioprotectant.

**Materials and methods**

1. **Preparation of $C_3$ by chemical synthesis:** The poor water solubility of fullerene seriously limits its application in biological study. We synthesized and purified the Carboxyfullerene $C_3$, a regioisomer of fullerene derivative containing three malonic acid groups per molecule according to the method of Lamparth and Hirsch [4]. The compound structure and molecular weight were confirmed by $^1$H-NMR, $^{13}$C-NMR, UV-vis and ESI.
2. **Cell culture：** AHH-1 human lymphoblastoid cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, Runnymede Malthouse Egham, UK) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg / ml of streptomycin at 37°C in a humidified, 5% $CO_2$ atmosphere in cell incubator.
3. **Administration of γ ray radiation:** $^{60}$Co theratron （Fyc－50H）was used to administer radiation with the radiation distance 75 cm, radiation dose rate 0.43 cGy/min, and the dose range from 1 to 8Gy according to the experiment design.
4. **Detection of cell survival rate after radiation exposure:** The exponentially growing AHH-1 cells were collected and subjected to γ ray radiation exposure. Then 100μl 0.4% trypan blue（Sigma USA） was added to the 100μl AHH-1 cell suspension ($4\times10^6$/L）), and cell counting was performed under microscope to number the dead and viable cells. Every sample was counted for 3 times and the data was resulted from repeated independent tests for 5 times. The cell survival rate was calculated as the following formula:

$$\text{cell survival rate} = \frac{\text{number of viable cells}}{\text{number of viable cells} + \text{number of dead cells}} \times 100\%$$

5. **Cell apoptosis analysis:** The AHH-1 cells were incubated with or without different concentration of $C_3$ in 24-well culture dish (Greiner Bio-one GmbH, Frickenhausen, Germany) in triplicate and subjected to radiation exposure. Cell apoptosis was detected with Annexin-V/PI kit (Invitrogen, Carlsbad, CA, USA) according to the manual with flow cytometer（Facscalibur BD）. Every experiment was repeated for 5 times, and cell apoptosis rate was calculated as following formula:

$$\text{apoptosis rate} = \frac{\text{Annexin - V single staining cells} + \text{Annexin - V / PI dual staining cells}}{\text{total cells}} \times 100\%$$

6. **The cytotoxicity of $C_3$：** The exponentially growing AHH-1 cells ($5\times10^5$/ml) were co-incubated with different concentration of $C_3$(0, 100, 200, 300 or 400 mg/L) for 48h and the cell survival rate was detected with trypan blue rejection test as described above.
7. **The protective activity of $C_3$ to γ ray radiation:** We investigated the radioprotective activity of $C_3$ on AHH-1 cell from three aspects.

7.1 **The influence of $C_3$ of different concentration on the survival rate and apoptosis of AHH-1 cells after radiation:** AHH-1 cells of exponential growth phase ($5\times10^5$/ml) were co-incubated with different final concentration of $C_3$ (0-600 mg/L) for 2h at $37^0$C with 5% $CO_2$ in saturated humidity. Then cells were subjected to 4Gy $^{60}$Co γ ray radiation exposure and continued culture for 48h followed by detection and analysis of cell survival rate and cell apoptosis rate.

7.2 **The influence of 200mg/L $C_3$ pre-treatment on AHH-1 cells radiated with different dose of $^{60}$Co γ ray:** The AHH-1 cells were divided into three groups: the control group (without radiation or $C_3$ treatment), the single radiation group (only received different dose of radiation), and radiation + 200mg/L $C_3$ group (received 200mg/L $C_3$ and followed by 1, 2, 4 or 8 Gy γ ray radiation). Then cells were continued culture for 48h after radiation exposure and subjected to analysis of cell survival rate and cell apoptosis rate.

7.3 **The influence of $C_3$ administration timing on the radioprotective effects:** The AHH-1 cells were administered with $C_3$ to a final concentration 200mg/L at different time point: 48, 24, 12, 8, 2, 0h before 4Gy radiation exposure and 0, 0.5, 2h after exposure, and continued culture for 48h， cell survival rate were examined as described.

8. **Statistical analysis:** The data are expressed as mean± SEM（$\bar{x} \pm s$）. Student's t-test was used, and *p* values less than 0.05 were considered significant.

## Results

1. **The cytotoxicity of $C_3$：** AHH-1 cells ($5 \times 10^5$/ml) were co-incubated with different concentration of $C_3$ for 48h and the cell survival rate was detected with trypan blue rejection test. The results showed $C_3$ had little influence on cell viability in our designed dose range (0-400 mg/L). The cell survival rate of 400mg/L $C_3$ group was 98.4±6.5%, which was similar to that of control group, indicating this compound is of low toxic effect on the AHH-1 viability (figure 1).

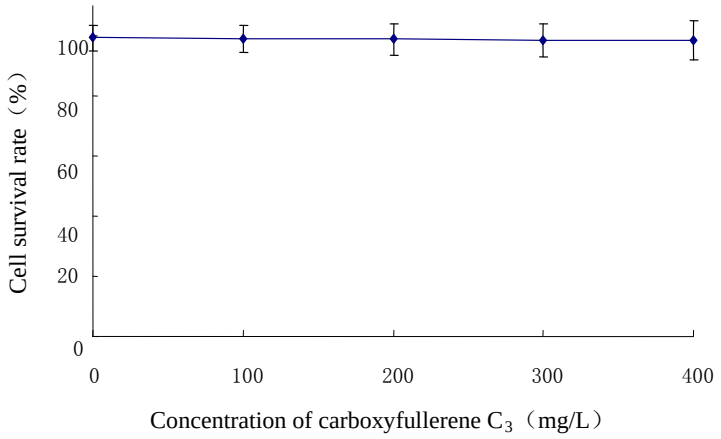


**Figure 1 The influence of $C_3$ on the cell survival rate of AHH-1**

2. **The protective activity of different concentration of $C_3$ on AHH-1 cells radiated with 4Gy γ ray:** The results showed $C_3$ had good radioprotection effects on AHH-1 against γ ray radiation. When the concentration was 10 mg/L,

$C_3$ showed protective effects to AHH-1 cell, which was enhanced with the increase of $C_3$ concentration. When the final concentration reached 400mg/L, the cell survival rate (91.2±4.9%) was similar to that of non-irradiated control cell (*P>0.05*) and significantly higher than that of single radiation group(67.6±5.4% *P <0.01*). While the irradiation induced apoptosis and death rate of $C_3$ treatment groups were significantly decreased in a dose dependant manner compared to that of single radiation group cells（*P <0.01*，Figure 2 and Table 1）.When the final concentration of $C_3$ reached over 200mg/L, the apoptosis rate was below 11.63±0.55%, which was of statistical significance compared with that of single radiation group(26.30±0.74%) (*P<0.01*, Table 1）.

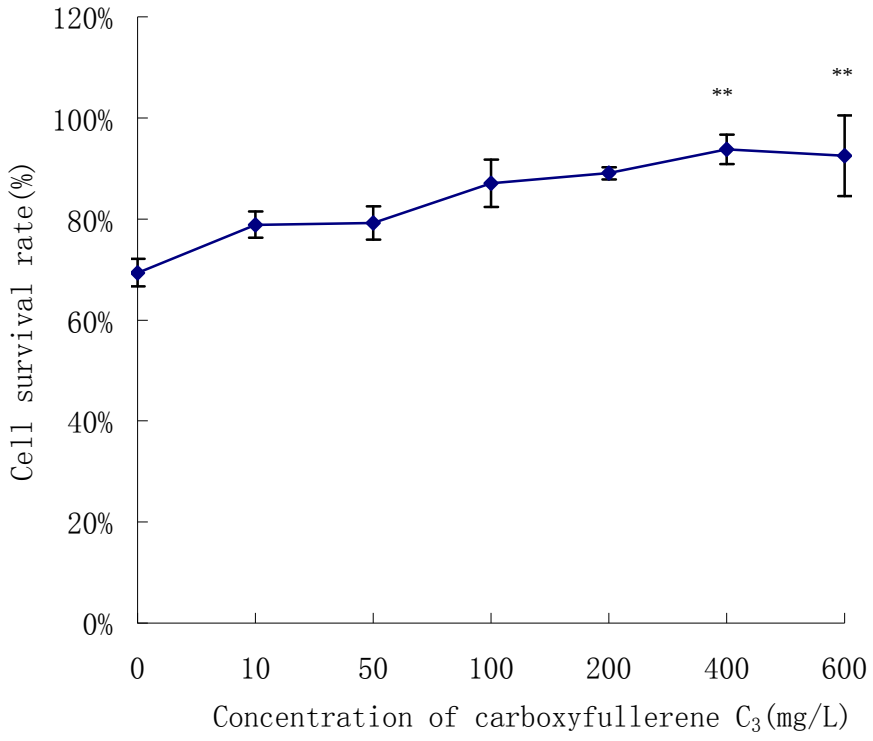


**Figure 2 The survival rate of AHH-1 cell co-incubated with different concentration of $C_3$ after 4Gy γ ray radiation**

** ***P<0.01*** **Vs single radiation group (without $C_3$ and received radiation)**

**Tab.1 The apoptosis rate of AHH-1 cells pre-treated with different concentration of $C_3$ after radiation**

| | $C_3$ concentration （mg/L） | | | | | |
|---|---|---|---|---|---|---|
| Apoptosis rate(%) | 0** | 10 | 50 | 100 | 200 | 400 |
| | 26.30±1.74 | 17.18±2.68* | 13.65±1.44* | 12.63±2.79* | 10.49±1.53* | 11.63±1.55* |

** **Single radiation group: cells received γ ray radiation and without $C_3$ pretreatment.**

* ***P<0.01*** **Vs the single radiation group.**

3. **The radioprotective effects of $C_3$ to AHH-1 cells radiated with different dose of γ ray:** The AHH-1 cells of exponential growth phase were divided into control group, single radiation group and radiation + $C_3$ group. The cells of radiation group were given different dosage of γ ray radiation（1, 2, 4 or 8Gy）; The radiation+ $C_3$ group was incubated with 200mg/ L $C_3$ which was administered 2h before radiation. We found $C_3$ pretreatment significantly decreased the apoptosis rate （*P<0.01*，figure 3）and increased the cell survival rate after radiation, particularly in the 200mg/ L $C_3$ + 4 or 8Gy radiation group（*P<0.01*，figure 4）.

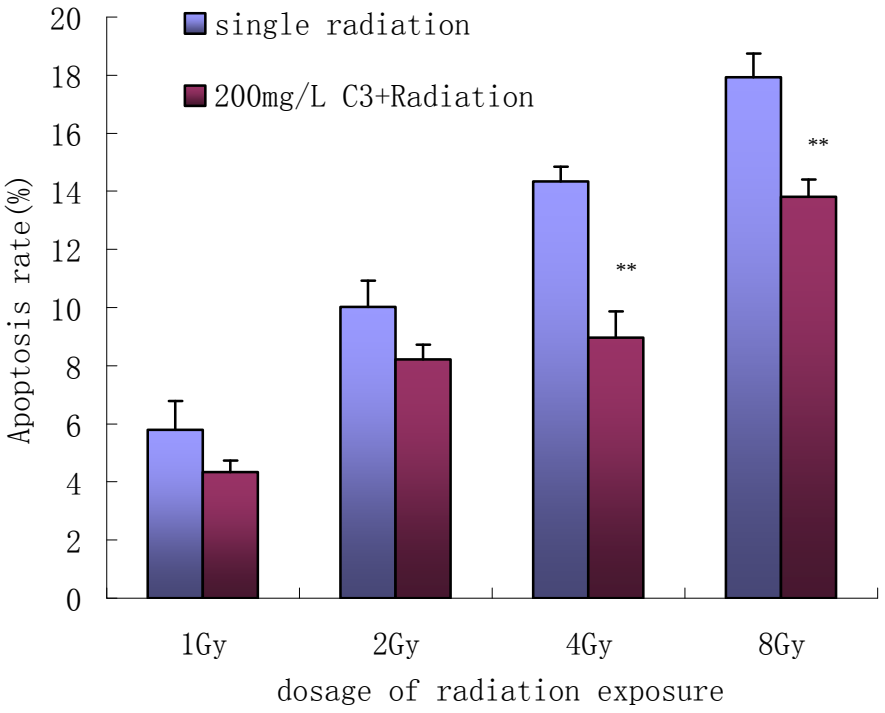


**Figure 3 The apoptosis rate of AHH-1 cells induced by different dose of radiation with or without $C_3$ pretreatment**

** ***P<0.01*** **Vs single radiation group.**

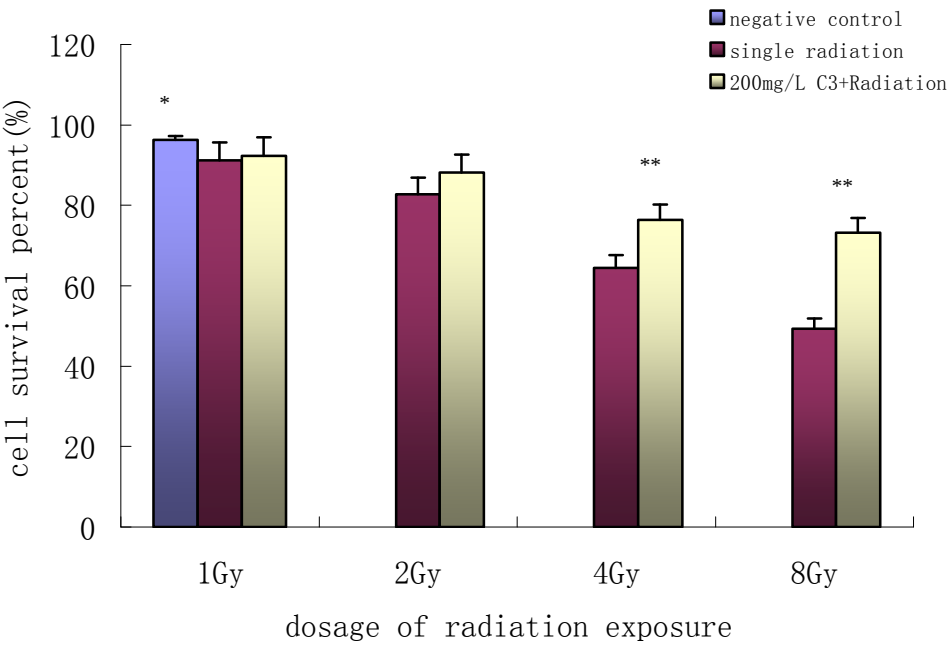


**Figure 4 The effect of $C_3$ on AHH-1 cell viability after different dose of radiation**

***negative control group: cells received no radiation and no $C_3$**

**** *P<0.01* Vs single radiation group**

4. **The effect of administration timing on the radioprotective ability of $C_3$ :** $C_3$ (final concentration 200mg/L) were administered to AHH-1 cells at different time points(48, 24, 12, 8, 2, 0h pre-radiation and 0, 0.5, 2h post-radiation). After continued incubation for 48h, trypan blue rejection test, Annexin-V/PI staining and flow cytometry were performed to detect the cell survival rate and apoptosis. The results showed $C_3$ can increase the cell survival rate after radiation in different extent （figure 5）. Administration of $C_3$ before radiation exposure showed better radioprotective effects and the interval time between $C_3$ administration and radiation exposure was closely related to the radioprotective effects. The cell survival rate in 24h pre-radiation $C_3$ group was markedly increased compared to the single radiation group and the most effective group was that administered 0h before radiation with a cell survival rate 91.5%, which was similar to that of control group( without radiation, 95.3%). The apoptosis rate of the 0h before radiation group (11.67%) was also significantly lower than that of single radiation group( 18.52%, $P < 0.01$, data not shown）.

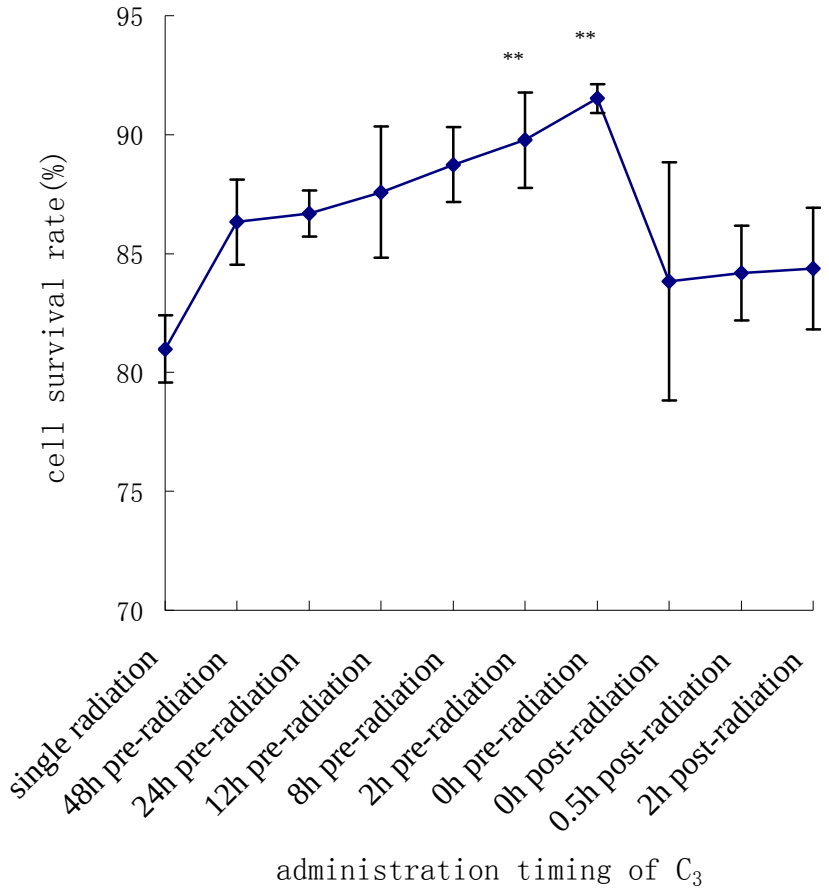


**Figure 5 The administration timing of $C_3$ on its radioprotective effect**

** ***P>0.05*** **Vs control group (without radiation).**

## Discussion

With the increasingly extensive exploitation of nuclear energy, its sever damages have attracted more and more concern in the world. Current multidisciplinary research in the field of radioprotection involves all aspects of basic and clinical research ranging from the subatomic mechanisms of free radical formation, macromolecular and intracellular radiation-induced alterations, biochemical and physiological homeostatic mechanisms [9]. Radioprotective agents, although widely studied in the past decades and including several thousand agents, have not provided an agent that conforms to all criteria of an optimal radioprotectant, including effectiveness, toxicity, availability, specificity and tolerance. Thus, it is necessary to find novel effective

radioprotectants, and free radical scavengers might be a promising approach.

Since the discovery of fullerene $C_{60,}$ its high-effective ability to scavenge free radicals has attracted extensive interests and become a hot spot in the biomedical research field [10, 11]. But the poor water solubility of fullerene limited its practical use, and several strategies have been developed to convert hydrophobic fullerene into water-soluble compounds by adding different groups of side chains to the fullerene molecules. Now some water-soluble fullerenes have become available, among which the carboxyfullerene $C_3$ is a promising active molecular because of its comparatively good water solubility and multifarious biological functions. It was firstly synthesized by Andreas Hirsch, and subsequent researches revealed $C_3$ has multifarious biological functions such as defending cell from oxidation stress, suppressing cell apoptosis, and attenuating the lung damages caused by ischemia-reperfusion etc and these functions are to some extent related to its characteristics as a free radical scavenger or an antioxidant agent [5, 11-13].

Since the indirect injury via free radicals is the main damage caused by ionizing radiation, it is rationally to postulate that Carboxyfullerene $C_3$ can protect cells from radiation induced damages. However, up to now the information on the radioprotective ability of $C_3$ is very limited. Fumelli C reported that carboxyfullerene can protect human keratinocytes from ultraviolet B damage via a mechanism interfering with the generation of reactive oxygen species from depolarized mitochondria [14]. We have been interested in the possibility that the potent innate free radical scavenging properties of $C_3$ could be harnessed for use in biological systems aiming at protecting cells from damages from ionizing radiation such as γ ray radiation.

In this study, we firstly evaluated the toxicity of $C_3$ to AHH-1 cells. The doses of $C_3$ used in this study ranged from 10 to 600 mg/L. All of these doses were non-toxic to AHH-1 cells as evidenced by the lack of acute toxicity associated death in $C_3$-treated group cells. The cell survival rate of 400mg/L $C_3$ treated AHH-1 cells reached 98.4±6.5%, which is similar to the level of control group（$P>0.05$）, indicating this compound is of low toxicity to the AHH-1 cells.

When different concentration of $C_3$ co-incubated with AHH-1 cells before γ-ray radiation, $C_3$ showed good radioprotective effects in a dose dependant manner. As low as 10 mg/L of $C_3$ has showed protection effects to AHH-1 cell as evidenced by elevated cell survival rate, which was further heightened with the increase of $C_3$ concentration. When the final concentration reached 400mg/L, the cell survival rate (91.2±4.9%) was similar to that of non-irradiated control cell (*P>0.05*) and significantly higher than that of single radiation group(67.6±5.4% *P <0.01*). The experiments on the radiation induced apoptosis rate further confirmed the protective effects of $C_3$ to AHH-1 cells. The radiation induced apoptosis and death rate of $C_3$ treatment groups were significantly decreased in a dose dependant manner compared to that of single radiation group cells（*P＜0.01*）. When the final concentration of $C_3$ reached over 200mg/L, the apoptosis rate was below 11.63±0.55%, which was of statistical significance compared with that of single radiation group(26.30±0.74%, *P<0.01*）.

In addition, we examined the effect of administration timing on its radioprotective ability. Administration of $C_3$ before radiation exposure showed better radioprotective effects than administered post-radiation exposure, and the interval time between $C_3$ administration and radiation exposure was closely related to the radioprotection effects. The cell survival rate in 24h pre-radiation $C_3$ group was markedly increased compared to the single radiation group and the most effective group was that administered 0h before radiation with a cell survival rate 91.5%, which was similar to that of control group( without radiation , 95.3%).

The good radiation protective ability of $C_3$ to AHH-1 cells may be attributed to its characteristic as a powerful free radical scavenger. It is well known that radiation induced injuries to cells are mainly caused by generous free radicals such as hydroxide radical, $O_2^{\cdot -}$ and aqueous electron etc. Timely eliminating these free radicals can mitigate or block various radiation-induced biological damages. Our previous study found 1000mg/L of $C_3$ can eliminated about 93% of hydroxide radical in the Fenton system. Dugan *et al.* also reported $C_3$ has excellent ability to scavenge both superoxide anions and hydroxyl radicals. Foley [15] used fluorescence microscopy

and radioactive labeling technique to observe the sub-cellular location of $C_3$ and found that $C_3$ can penetrate through cellular membrane and enter the cell. In our study, the pre-existed cellular $C_3$ through incubation of $C_3$ with AHH-1 cell can timely scavenge the radiation induced free radicals, thus protect cells against apoptosis or death caused by γ ray radiation. In addition, the cellular radioprotective effects of $C_3$ might also involved the anti-oxidation pathway, as was confirmed by Sameh [16] that $C_3$ can act like SOD, protecting organisms against peroxidate compounds through dismultation.

As for the better protective effects of pre-radiation administration than that of post-radiation administration, we speculate that it is related to the life-time of free radicals and the metabolism of $C_3$. Since half-life time of most free radicals induced by radiation are very short (measured by seconds), if $C_3$ is administered post-radiation, most of free radicals have already caused tremendous damages, which would degrade the protective activity of $C_3$. On the other hand, when $C_3$ enter the cells, it will be metabolized and removed in a certain time (about 48h). Therefore, if administered too early before radiation, most of $C_3$ would be eliminated from cells when free radicals are produced by radiation. To achieve good radioprotective effects, $C_3$ should be administered before radiation exposure with a suitable interval time.

Taken together, when given prior to irradiation, Carboxyfullerene $C_3$ has good radioprotective effects to AHH-1 cells, which was evidenced by increased survival rate and decreased radiation induced apoptosis rate. In the effective drug concentration range of this study, $C_3$ had little toxicity to AHH-1 cell which suggest that $C_3$ have the potential to be a novel promising radioprotectant. Our study provides an experimental basis for $C_3$ to be exploited as an effective radioprotectants with low toxicity.

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