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Kojic Acid Protects C57BL/6 Mice from Gamma-irradiation Induced Damage

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Abstract

The radioprotective effects of a single administration of kojic acid (KA) against ionizing radiation were evaluated via assessment of 30-day survival and alterations of peripheral blood parameters of adult C57BL/6 male mice. The 30-day survival rate of mice pretreated with KA (75 or 300 mg/kg body weight, KA75 or KA300) subcutaneously 27 h prior to a lethal dose (8 Gy, 153.52 cGy/min) of gamma irradiation was higher than that of mice irradiated alone (40% or 60% vs 0%). It was observed that the white blood cell (WBC) count/the red blood cell (RBC) count, haemoglobin content, haematocrit and platelet count of mice with or without KA pretreatment as exposed to a sub-lethal dose (4 Gy, 148.14 cGy/min) of gamma irradiation decreased maximally at day 4/day 8 post-irradiation. Although the initial WBC values were low in KA300 or WR-2721 (amifostine) groups, they significantly recovered to normal at day 19, whereas in the control group they did not. The results from the cytotoxicity and cell viability assays demonstrated that KA could highly protect Chinese hamster ovary (CHO) cells against ionizing radiation with low toxicity. In summary, KA provides marked radioprotective effects both in vivo and in vitro.

Keywords: Kojic acid - radioprotection - C57BL/6 mice - Chinese hamster ovary cells - gamma irradiation damage

Introduction

KA is a naturally available fungal metabolic product produced by many species of Aspergillus, Penicillium, and Acetobacter (Parrish et al., 1966; Bentley, 2006), and is widely existed in fermented foods, including miso, soy sauce, and rice wine, which are usually consumed in Japan. It is reported that KA can protect skin from pigmentation (Chisvert et al., 2010; Kwak et al., 2011) and can suppress enzymatic browning of fruits, raw shrimps and crabs (Nohynek et al., 2004), and is widely utilized in cosmetics and food processing areas. Moreover, recent literature has examined potential pharmacological effects, including radioprotective (Emami et al., 2007; Hosseinimehr et al., 2009), anti- HIV reverse transcriptase (Tanaka et al., 2009), anti-diabetic (Wei et al., 2011) and anti-convulsant (Aytemir et al., 2010) for KA and its derivatives apart from the above properties. Emami S. et al. studied the radioprotective effects in male NMRI mice for KA with pretreatment 24 h before exposed to a lethal dose of gamma irradiation (Emami et al., 2007). Based on their findings, we carried out in vivo and in vitro experiments to further confirm the radioprotective effects of KA.

Nowadays, there are a great variety of radioprotectors in our daily lives, including synthetic (Cassatt et al., 2002), anti-oxidant (Samarth et al., 2008; Andrievsky et al., 2009; Cressier et al., 2009; Dhaker et al., 2011), naturally occurring radioprotectors (Hosseinimehr et al., 2003; Samarth et al., 2004; Byron et al., 2008; Hosseinimehr, 2010; Sebastià et al., 2011; Sebastià et al., 2013), and immunomodulators (Landauer et al., 2003) as well. Although WR-2721 (amifostine) is the only radioprotector that has been approved by the US Food and Drug Administration at present, it has some severe adverse effects including nausea, vomiting, lethargy, and hypotension with large toxicity (Landauer et al., 1987; Koukourakis et al., 2000). Furthermore, amifostine must be administrated by the intravenous route, which restricts its clinical usage in patients. For these reasons, the search for the radioprotectors which are more effective, less toxic, and have more acceptable route and frequency of administration is much more important and necessary. In this research, the role of KA in vivo and in vitro to protect against gamma irradiation was investigated. Different from that of previous studies by Emami S. et al, C57BL/6 mice were used and the time for KA pretreatment was

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Table 1. The 30-day Survival of Mice Pretreated with KA 27 h or WR-2721 1 h Before Exposed to a Lethal Dose (8 Gy, 153.52 cGy/min) of Gamma Irradiation

| Group | Mortality on 30 days post-irradiation | Total Survival | Statistical Test | Chi-square | Log Rank | Breslow | Tarone-Ware |
|-------|-------------------------------------|---------------|------------------|------------|----------|---------|------------|
|       |                                     |               |                  | F(x)       |          |         |            |
|       |                                     |               |                  | p<0.0001   | p<0.0001 | p<0.0001 | p<0.0001   |
|       |                                     |               |                  | p=0.0009   | p=0.0014 | p=0.0074 | p=0.0002   |
|       |                                     |               |                  | p=0.0033   | p=0.0072 | p=0.0002 | p=0.0001   |
|       |                                     |               |                  | p=0.0017   | p=0.0017 | p=0.0002 | p=0.0001   |

Group 1, 27 h of KA 27 h before irradiation; Group 2, 1 h of WR-2721 1 h before irradiation; Group 3, 27 h of KA 1 h before irradiation; Group 4, 27 h of WR-2721 1 h before irradiation. Then, blood was collected at day 1, 4, 8, 19 post-irradiation. For the survival studies, 80 mice used in this experiment were divided into four groups (Irradiation group, KA75 group, KA300 group, WR-2721 group) of twenty mice each ad libitum.

Mice in KA 75 and KA300 groups were administered single subcutaneously (s.c.) doses of KA (75 mg/kg (0.5 mM/kg) and 300 mg/kg (2 mM/kg)) 27 h prior to irradiation in the 30-day survival investigations. In addition, effects of KA on the haematological parameters of mice exposed to 4 Gy sub-lethal dose of gamma irradiation were assessed. In vitro experiments, we examined the cytotoxicity of KA and its effects on cell viability as CHO cells exposed to ionizing radiation. Our results indicated that KA may play an important role in reducing the cell death induced by ionizing radiation and increasing the survival rate of mice as exposed to gamma-irradiation and may have great potential as a radioprotector.

**Materials and Methods**

**Drugs and reagents**

Kojic acid with a purity greater than 99% was supplied by Shandong Jinan Hengjia Medicine Chemical Development Co. Ltd. (Shandong, Jinan, China). WR-2721 was provided by Schering-Plough (China) Ltd. (Beijing, China). RPMI-1640 medium and FBS were purchased from Gibco (California, USA). Dimethyl sulfoxide (DMSO) was obtained from Sinopharm Chemical Reagent Co. Ltd. China, and Thazoyl Blue Tetrazolium Bromide (MTT) from Amresco (Solon, OH, USA).

**Mice**

Male C57BL/6 mice (seven to nine week old, weighing 22-24 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and housed in the Experimental Animal Center of the Institute. Mice were provided with food and water ad libitum. The animals were housed in a polypropylene cage containing the sterile serrago. Meanwhile, mice were maintained under the controlled conditions of a 12 h light/dark cycle, temperature (22±2°C) with 54% humidity. This study was approved by the institutional ethics committee of Beijing Institute of Radiation Medicine.

**Irradiation**

For in vivo experiments, all the animals were placed in well-ventilated perspex cages, and were performed with a single whole-body exposure of the mice to 8 Gy or 4 Gy gamma irradiation via a cobalt-60 gamma radiation source (REVIISS, UK). The source-to-skin distance was 400 cm with a dose rate of 153.52 or 148.14 cGy/min at room temperature (23±2°C). For in vitro experiments, cells were seeded in 96-well plates, and then KA solution was applied to cells prior to gamma irradiation, and the dose rate for cells was between 150 and 153.14 cGy/min.

The-30-Day Survival studies

For the survival studies, 80 mice used in this experiment were divided into four groups (Irradiation group, KA75 group, KA300 group, WR-2721 group) of twenty mice each ad libitum.

Mice in KA 75 and KA300 groups were administered single subcutaneously (s.c.) doses of KA (75 mg/kg (0.5 mM/kg) and 300 mg/kg (2 mM/kg)) 27 h prior to a lethal dose of 8 Gy whole-body gamma irradiation at a dose rate of 153.52 cGy/min, respectively. Mice in Irradiation group only received an equal volume of sterile double distilled water 27 h before exposed to 8 Gy of gamma irradiation, subcutaneously. And mice in WR-2721 group were subjected to a s.c. injection of amifostine 214 mg/kg (1 mM/kg) 1 h prior to a lethal dose of gamma irradiation. Then all the animals were observed and recorded daily up to 30 days for the mortality and behavioral toxicity, and the body weight of the animals were also recorded every other day.

**Haematological studies**

For haematological studies, 40 mice were divided into five groups (Normal group, without any treatment; Irradiation group (Control), only treated with 4 Gy of gamma irradiation; KA75 group, 75 mg/kg body weight of kojic acid+4 Gy of gamma irradiation; KA300 group, 300 mg/kg body weight of kojic acid+4 Gy of gamma irradiation; WR-2721 group, 214 mg/kg body weight of WR-2721+4 Gy of gamma irradiation) with 8 mice in each group at random. All the animals were given adaptive feeds for four days at our animal center. Blood was collected from the tail vein in a vial containing anticoagulant agents at day 4. Afterwards, except for Normal group and Irradiation group, mice in the other three groups were subjected to single doses of KA/WR-2721 at 27 h/1 h before exposed to gamma irradiation (4 Gy, 148.14 cGy/min). Then, blood was collected at day 1, 4, 8, 19 post-irradiation. The values for the...
Cell culture, cytotoxicity, and cell viability assay

CHO cells (ATCC, USA) were incubated in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, and maintained at 37°C in a 5% CO₂ incubator. For cytotoxicity assay, CHO cells were seeded in 96-well plates at a density of 2500 cells per well in 180 μl medium. Then, 20 μl of CHO stock solutions (KA powder was dissolved in sterile double distilled water 27 h prior to 8 Gy whole-body gamma irradiation. Mice in WR-2721 group were subjected to 214 mg/kg body weight of WR-2721 subcutaneously 1 h prior to 8 Gy of gamma irradiation (n=20 per group) were added with 20 μl MTT (5 mg/mL), which was dissolved in sterile Phosphate Buffered Saline (pH 7.4) and filtered through 0.22 μm microporous membrane) were added to each well to make sure KA at the final concentrations of 0, 0.1, 1, 10, 100, 1000, 1500, 2500 μg/mL. After cultured for 72 h, each well of the cells was added with 20 μl MTT (5 mg/mL), which was dissolved in sterile Phosphate Buffered Saline (pH 7.4) and incubated for 4 h at 37°C. Then the supernatant was discarded and 200 μl DMSO was added to each well. The mixture was sufficiently oscillated in the dark place for 15 min. The optical density values of wells were measured in Multiskan MK3 ELISA Reader (Thermo, USA) at 492 nm. Cell viability assays were also analyzed by using MTT assay, in which CHO cells were cultured at a density of 2500 cells/well in 96-well plates and pretreated with single different concentrations (0-100 μg/ml) of KA for 1.5 h and then exposed to 6 Gy gamma irradiation at the dose rate of 150.95 cGy/min. The percentage of inhibition for the cytotoxicity and viability for cells was calculated according to the following formulas: Inhibition%= (C-T)/C×100%; Viability%=T/C×100%, where T and C stand for the absorbance of treatment group and the control group (0 μg/ml, KA), respectively.

Statistical analysis

All statistical analyses were carried out by SPSS version 13.0 for Windows. Only in vitro experiments are expressed as the means±SD of three independent experiments. Thirty days observation period were analyzed by using the Kaplan-Meier equation. The survival rate of mice in different groups was compared using two-sample test for proportions with Fisher’s Exact Test, Log Rank Mantel-Cox, Breslow, and Tarone-Ware statistics (Table 1). LSD’s Post Hoc Test was performed for multiple group comparison, p < 0.05 were considered significant and p < 0.01 highly significant.

Results

The-30-Day Survival studies

The 30-day survival rates of different groups of mice irradiated at a lethal dose of 8 Gy are shown in Figure 1 and Table 1. It was observed that 20 mice in irradiation group, injected s.c. with a single sterile distilled water 27 h before exposed to gamma irradiation, totally died at day 20 (0% Survival). In contrast, the administrations of two doses of kojic acid (75 and 300 mg/kg body weight) at 27 h prior to irradiation both kept mice alive at rates of 40% and 60% during the 30-day observation period. The 30-day survival rate of WR-2721 pre-treated mice was the highest (95%) among the 4 groups.

During the period of 30 days, the clinical symptoms of radiation sickness were investigated in mice. From day 6 after exposure, the main symptoms of hypoaactivity, epilatonia, and lethargy could be observed. Later, humpback and convulsion were sometimes observed in 11 days. Twenty one days after exposure to gamma irradiation, the symptoms of all mice started reverting to normal.

Changes of the body weight of mice in different groups after exposed to irradiation have been shown in Figure 2, for which, the animals began to decline two days after irradiation. And mice pretreated with
Table 2. Variations in Peripheral Blood Parameters of Mice Pretreated with WR-2721 or KA as Exposed to 4 Gy of Gamma Irradiation (\(\chi^2\), n=8)

| Post-Irradiation Intervals | Group    | WBC (\(\times 10^3/\text{L}\)) | RBC (\(\times 10^5/\text{L}\)) | Hb (g/L) | HCT | PLT (\(\times 10^3/\text{L}\)) |
|----------------------------|----------|-------------------------------|-------------------------------|---------|-----|-------------------------------|
| 1 day                      | Normal   | 14.20±4.58                   | 9.86±0.37                     | 182.60±4.98 | 0.518±0.01 | 1326±185                     |
|                            | Control  | 3.00±0.14***                 | 10.75±0.80                    | 183.20±9.12 | 0.511±0.04 | 1310±137                     |
|                            | KA75     | 6.15±2.65***                 | 10.49±0.57                    | 190.00±6.56 | 0.529±0.03 | 1311±89                      |
|                            | KA300    | 7.57±1.77***                 | 10.98±0.34*                   | 201.80±7.95*** | 0.555±0.02* | 1364±49                      |
|                            | WR-2721  | 3.77±0.45***                 | 10.27±0.41                   | 185.20±5.40 | 0.511±0.03 | 1326±75                      |
| 4 days                     | Normal   | 14.10±0.47                   | 9.94±0.74                     | 189.50±10.82 | 0.500±0.02 | 1590±59                      |
|                            | Control  | 2.85±0.07***                 | 8.32±0.87***                  | 161.7±5.38*** | 0.424±0.03*** | 1525±230                     |
|                            | KA75     | 4.07±1.95***                 | 9.10±0.14                    | 174.50±3.53** | 0.466±0.02** | 1556±178                     |
|                            | KA300    | 4.25±0.07***                 | 9.42±0.41*                    | 171.00±2.64** | 0.481±0.02** | 1633±40                      |
|                            | WR-2721  | 4.40±1.98***                 | 9.55±0.38**                   | 178.00±5.55** | 0.477±0.03** | 1759±88*                     |
| 8 days                     | Normal   | 14.00±2.61                   | 9.82±0.80                     | 171.67±8.39 | 0.513±0.03 | 1525±208                     |
|                            | Control  | 4.12±1.26***                 | 7.31±0.84***                  | 122.33±3.21*** | 0.370±0.02*** | 505±85***                     |
|                            | KA75     | 6.12±1.95***                 | 8.10±0.61***                 | 132.75±4.27*** | 0.395±0.03*** | 597±67***                     |
|                            | KA300    | 6.80±0.71***                 | 8.77±0.63***                 | 150.17±3.76*** | 0.424±0.03*** | 697±311***                    |
|                            | WR-2721  | 7.30±1.55***                 | 9.73±0.43*                   | 165.20±8.79** | 0.487±0.04** | 848±79***                     |
| 19 days                    | Normal   | 14.30±3.61                   | 9.87±0.99                     | 176.60±5.64 | 0.480±0.02 | 1548±178                     |
|                            | Control  | 9.83±2.95*                   | 9.00±0.79                    | 162.67±7.02** | 0.450±0.06 | 1366±81                      |
|                            | KA75     | 10.40±0.62*                  | 9.92±0.56*                   | 158.71±4.89*** | 0.471±0.02  | 1300±63*                     |
|                            | KA300    | 14.30±2.03*                  | 9.97±0.51*                   | 168.20±7.19** | 0.489±0.04 | 1458±148                     |
|                            | WR-2721  | 16.5±4.45**                  | 9.61±0.68                    | 157.50±7.77** | 0.453±0.03 | 1349±98*                     |

Normal group, with no treatment; Control group or Irradiation group, 4 Gy of gamma irradiation+sterile double distilled water; WR-2721 group, 214 mg/kg body weight of WR-2721+4 Gy of gamma irradiation; KA75 group, 75 mg/kg body weight of kojic acid+4 Gy of gamma irradiation; KA300 group, 300 mg/kg body weight of kojic acid+4 Gy of gamma irradiation. *p < 0.05, **p < 0.01, ***p < 0.001 compared with Normal group; †p < 0.05, ‡p < 0.01 compared with Control group. Each value represents means±SD.

WR-2721/KA started recovering at day 6/day 14 post-irradiation. It was also observed that the body weight of mice decreased sharply up to 12 days and 14 days after exposed to gamma irradiation which was in accordance with concentrated death rate of mice (from day 12 to day 16 post-irradiation).

Haematological Examinations

The WBC counts of mice decreased markedly at day 1 (p < 0.001) following a sub-lethal dose (4 Gy) of gamma irradiation in treatment groups (Irradiation or Control groups, KA75, KA300, and WR-2721) at all intervals in comparison with the normal group, with a maximum decline at day 4 (Table 2). Later, the number of such cells in mice of KA300 or WR-2721 pretreated groups elevated gradually and reached to the normal level at day 19. However, mice in Irradiation group and KA75 group did not.

The RBC counts of animals irradiated alone did not show any significant (p>0.05) change at day 1, however, in mice pretreated with KA (300 mg/kg body weight), it was found a significant (p < 0.05) increase as compared with the normal group. At day 4, in mice irradiated alone, the RBC count was found significantly (p < 0.001) lower than the normal, and treatment groups were also observed slight declines but did not show significant (p > 0.05) differences, however, WR-2721 and KA300 groups were found significant differences as compared with the control group. Maximum decreases were observed in treatment groups at day 8. Afterwards, the treatment groups recovered normal at day 19.

Likewise, the hemoglobin (Hb) contents and hematocrit (HCT) in treatment groups did not show any significant change at day 1, and the Hb contents in KA300 group were higher than in the normal group, which was similar to the RBC counts. The maximum declines were observed at day 8 (Table 2) and in KA300 and WR-2721 groups/KA75 and WR-2721 groups, the Hb contents/HCT were found significant differences as compared with the control. Afterwards, all treatment groups nearly reached normal at day 19 post-irradiation (Table 2).

Cytotoxicity

The cytotoxicity for a large range of concentrations of KA in CHO cells in different intervals were measured using MTT assay. As shown in Figure 3A, CHO cells pretreated with KA did not show any change when the final concentration of KA was lower than 100 μg/ml, and the cytotoxicity of KA could be detected in its high concentrations and especially when the cultured time reached 72h, the IC50 value was estimated as 1254.8 μg/ml (Figure 3B).

Cell viability

As CHO cells were exposed to gamma irradiation...
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Discussion

Kojic acid is a very important organic acid produced by many species of Aspergillus, Penicillium, and Acetobacter, and is widely used in cosmetics and food additives. It has been indicated that kojic acid has properties of radioprotectors (Emami et al., 2007; Hosseinimehr et al., 2009) and antioxidants and can stimulate the neutrophilic granulocyte phagocytosis and lymphocyte proliferation (Niwa and Akamatsu, 1991).

In the present investigation, C57BL/6 mice, the most optimal strain mouse, in radiation injured animal experiments were used. In the 30-day survival evaluation, we found that a single s.c. injection of KA (KA300 group) 27 h prior to a lethal dose of gamma irradiation could elevate the survival rate of mice up to 60% during a period of 30 days, and the efficacy on KA could comparable to that reported by Emami et al., while mice irradiated alone (Irradiation group) wholly died within 20 days, which demonstrated that KA has a marked protective effect on irradiation-induced death in C57BL/6 mice (Figure 1). Although KA is not as strong as WR-2721 in radioprotective efficacy, the former is more useful in prolonging the action period between body and drug, and is less disadvantage than the latter in safety.

For haematological examinations, it was observed that all haematological parameters of mice with or without treatment 27 h before exposed to a sub-lethal dose (4 Gy) of gamma irradiation compared with the normal group were decreased. However, these parameters were increased significantly as mice pretreated with KA or WR-2721 compared with the irradiation group, especially the KA300 or WR-2721 group.

Exposure of animals to ionizing radiation can induce long-term hematopoietic cells damage, which is mainly the self-renewal damage for hematopoietic stem cells (HSCs) (Chua et al., 2012). Wang et al. (2006) found C57BL/6 mice exposed to a sub-lethal dose of gamma irradiation induced a persistent reduction in LKS+ HSCs. For vulnerable tissues or organs including bone marrow, gamma irradiation can generate some aspects of pathological changes in cellular and molecular levels and cause DNA fragmentation (Sutherland et al., 2002; Suman et al., 2012; Floratou et al., 2012), chromosome aberration, hemopoietic function disturbance, and ultimately result in bone marrow depression (Yankelevitz et al., 1991), microcirculation disturbance (Carbonneau et al., 2012) and the decline in peripheral blood cells. Therefore, bleeding and infections are the main reasons for the death of acute radiation sickness (Williams and McBride, 2011).

The WBCs are highly sensitive to radiation, as shown in Table 2, the value of the WBC counts in mice irradiated alone decreased gradually at day 1 post-irradiation, with a marked decline at day 4 and then started reverting, however, failed to reach the normal value at day 19. In contrast, mice in KA300 group reverted to normal at day 19, which demonstrates that KA provides protection to the WBCs. Thus, it can be indicated that WBC is the most foundational index to radiation damage.

In the present study it was observed that the RBC counts, Hb contents, and HCT of mice in all groups
exposed to a sub-lethal dose (4 Gy) of gamma-irradiation at day 1 post-irradiation did not show any decline compared with the normal value, which mainly because erythrocytes can live for 120 days and the mature RBCs are very radioresistant owing to their lack of DNA and apoptotic machinery (Puchała et al., 2004). The RBC counts, Hb contents, and HCT of mice pretreated with KA (300 mg/kg body weight) reverted to normal at day 14, but mice irradiated alone did not, which indicates KA provides protection to RBCs and hemoglobin contents, and protects against anaemia induced by ionizing radiation.

There are many aspects of radiation effects on platelets, which not only affect the production and consumption, but also influence the function. Platelets can live for 9-10 days, meanwhile, mature megakaryocytes which are not damaged by irradiation still cause thrombocytopenia in the initial stage. Thus, the values of platelets decrease slowly within 8 days post-irradiation and afterwards, hematopoietic progenitor cells decline sharply and result in the generous reduction to platelets.

Apart from in vivo investigations, effects of KA on the cytotoxicity and radiation protection in vitro were also performed. For cytotoxicity studies, it was found that KA has no influence on CHO cell at its concentrations less than 100 μg/ml (Figure 3), but inhibits the cells when the concentrations reached a high range (IC50 = 1254.8 μg/ml, 72 h). The cytotoxicity of KA at high concentrations might be related to its disturbance to the pH of culture medium. For cell viability assays, we found that KA provides a radioprotective effect to CHO cells in a range of concentrations lower than 100 μg/ml, and 1.5 h pretreatment is optimal for radioprotective effect (data not shown).

Taken together, it was proven that KA exerts its radioprotective effects on lethal or sub-lethal irradiation induced damages in mice in this study, and KA can protect cultured cells from irradiation. In conclusion, KA has great potential as a new class of radioprotector with lower toxicity, although the mechanisms of action for kojic acid need to be further elucidated.

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