Dark tea extract mitigates hematopoietic radiation injury with antioxidative activity

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ABSTRACT

The hematopoietic system is widely studied in radiation research. Tea has been proved to have antioxidative activity. In the present study, we describe the protective effects of dark tea extract (DTE) on radiation-induced hematopoietic injury. DTE administration significantly enhanced the survival rate of mice after 7.0 and 7.5 Gy total body irradiation (TBI). The results showed that DTE not only markedly increased the numbers and cloning potential of hematopoietic cells, but also decreased DNA damages after mice were exposed to 6.0 Gy total body irradiation (TBI). In addition, DTE also decreased the levels of reactive oxygen species (ROS) in hematopoietic cells by inhibiting NOX4 expression and increasing the dismutase, catalase and glutathione peroxidase in livers. These data demonstrate that DTE can prevent radiation-induced hematopoietic syndromes, which is beneficial for protection from radiation injuries.

Keywords: ionizing radiation; hematopoietic cells; mice; dark tea extract

INTRODUCTION

With the popular application of ionizing radiation, the need for radiation protection and radiation injury therapy has increased. Only amifostine has been approved by the US FDA for use in the clinic as a radiation protector, its side effects and limitations could not meet the requirements for the wide use in radiation protection [1]. Therefore, it is necessary to explore new methods for providing radiation protection [2].

Tea is a kind of popular beverage and has been consumed for thousands of years. Depending on the degree of fermentation and the manufacturing process, China’s tea can be classified into four categories: non-fermented (green tea), semi-fermented (oolong tea), fully-fermented (black tea) and post-fermented (dark tea) [3]. The protective effects of both black tea and green tea on radiation damages have been reported extensively [4, 5]. Some extracts such as tea polyphenols and epigallocatechin gallate have also been proved to mitigate radiation injuries [6, 7].

Dark tea is one of the traditional and unique tea products made in China and limited to the areas of Yunnan, Hunan, Hubei, Sichuan and Guangxi provinces. The fermentation steps in the process of making dark tea involve more microorganisms, which results in changes in the chemical composition of the tea of polysaccharides, polyphenol, theabrownin and caffeine [8]. Tea polyphenol oxidative polymerization reaction (caused by polyphenol oxidase) led to a decrease in the content, composition and proportion of catechins, an obvious change that the 8-C-N-ethyl-2-pyrrolidinone in the dark tea had significant antioxidative activity [9]. Recently, Chen showed that free amino acids were accumulated in dark tea by the proteolysis of chloroplast proteins [10]. A number of studies report that dark tea has various health benefits, such as anti-hyperlipidemia, anti-obesity, antibacterial action, inhibition of fat deposition, and so on [11–16]. Cheng showed that water extract of dark tea had in vitro antioxidant activity [17]. Wang reported that the 8-C-N-ethyl-2-pyrolidinone in the dark tea had significant antioxidative activity [14]. However, protective effects of dark tea in the area of irradiation injury have not been reported.

In this study, we investigated the effects of dark tea extract (DTE) on hematopoietic radiation injury. We first confirmed that DTE could elevate the survival rate of mice exposed to 7.0 and 7.5 Gy total body irradiation (TBI). Then we found DTE protected the hematopoietic system in mice exposed to 6.0 Gy TBI by decreasing the reactive oxygen species (ROS) levels and NOX4 expression. In addition, the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) in the liver were all elevated by DTE.
METHODS

All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Institute of Radiation Medicine (IRM), the Chinese Academy of Medical Sciences (CAMS). The animals were cared for in accordance with the dictates of the National Animal Welfare Law of China.

Reagents

The anti-mouse Ly-6A/E (Sca-1)-PE/Cy7, CD117 (c-kit), APC, biotin-conjugated CD5, CD4, CD8, CD45R/B220, Ly6G/Gr-1, CD11b, Ter-119, and APC/CY7-conjugated streptavidin antibodies were purchased from eBioscience (San Diego, CA, USA). The 2',7-dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Sigma-Aldrich (St Louis, MO, USA). The RPMI 1640 medium was purchased from Gibco (Grand Island, NY, USA). The BD Cytofix/Cytoperm buffer was purchased from BD Biosciences (San Diego, CA, USA). Methylcellulose M3434 was purchased from Stem Cell (Vancouver, BC, Canada). Fetal calf serum was purchased from Biological Industries (Kibbutz, Israel). The rabbit anti-H2AX antibody was obtained from Cell Signaling Technology (Danvers, MA, USA), the rabbit anti-NOX4 antibody from Proteintech (Wuhan, China) and the FITC-conjugated goat anti-rabbit antibodies from Abcam (Cambridge, MA, USA). Hei brick tea was purchased from Baishaxi Tea Industry (Hunan, China). Malondialdehyde (MDA), SOD, CATand GSHPx reagent kits were purchased from Nanjing Jiancheng Bioengineering (Jiangsu, China).

Animal studies

Male C57BL/6 mice weighing 18–22 g and 6–8 weeks of age were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China) and housed in the Institute of Radiation Medicine, CAMS. Animals were housed in the certified animal facility in the Institute of Radiation Medicine (IRM), CAMS. All procedures involving animals were reviewed and approved by the Animal Care and Use Committee (ACUC) of IRM (Permit Number 1523). For the survival study, irradiated mice were monitored once a day. When an animal met the definitive criteria for moribundity (abdominal breathing, inability to stand, or inability to right itself within 5 s when placed gently on its side), it was humanely euthanized at an early endpoint by cervical dislocation, in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals.

Water extract of dark tea

An extract was obtained from the dry, crushed tea leaves (100 g) using 1000 ml distilled water at 80°C in a water bath for 20 min. It was then filtered. The residues were extracted under the same conditions two further times. All the filtrate water was centrifuged to remove contaminants and the supernatant was lyophilized to dryness. The lyophilized material was used to conduct pharmacological experiments.

Irradiation and treatment

Irradiation was performed using a 137Cs source housed in an Exposure Instrument Gammacell-40 (Atomic Energy of Canada Lim, Chalk River, ON, Canada) at a dose rate of 1.0 Gy per min. After irradiation, the mice were returned to the animal facility for daily observation and treatment as described below. The mice were exposed to 7.0 and 7.5 Gy TBI in the survival experiments and 6.0 Gy TBI in the remaining experiments. The mice in the 7.0 Gy survival experiments were randomly assigned to five treatment groups: control (n = 10), vehicle + 7.0 Gy TBI (n = 12), 50 mg/kg DTE + 7.0 Gy TBI (n = 13), 100 mg/kg DTE + 7.0 Gy TBI (n = 13) and 200 mg/kg DTE + 7.0 Gy TBI (n = 12). The 75 mice in the 7.5 Gy survival experiments were randomly assigned to 5 treatment groups (n = 15): control, vehicle + 7.5 Gy TBI, 50 mg/kg DTE + 7.5 Gy TBI, 100 mg/kg DTE + 7.5 Gy TBI, and 200 mg/kg DTE + 7.5 Gy TBI. For the DTE treatment, the mice were administered DTE by gavage 14 times over the 14 days prior to irradiation and 7 times after irradiation. The control and 7.0 Gy TBI groups were treated with vehicle similarly to the procedure described for the DTE treatments. All the mice in the irradiated groups were irradiated 1 h after the last treatment. The 20 mice in the remaining experiments were randomly assigned to four treatment groups: control, 200 mg/kg DTE + 6.0 Gy TBI, vehicle + 6.0 Gy TBI, and 200 mg/kg DTE + 6.0 Gy TBI. The mice were treated as described above and were killed 9 days after exposure to irradiation.

Peripheral blood cell and bone marrow mononuclear cell counts

Blood samples were obtained from the orbital sinus after mice were exposed to 6.0 Gy TBI for 9 days. The bone marrow (BM) cells were flushed from mouse femurs with PBS after the mice were euthanized. The numbers of the various blood cell types and bone marrow mononucleated cells (BMNCs) were counted using a BD FACSCalibur flow cytometer (Becton, Dickinson, Franklin Lakes, NJ, USA) for 20 min at 37°C. The intracellular ROS levels were measured using a Methylcellulose M3434 methylcellulose medium. Colony-forming unit granulocyte–macrophage (CFU-GM), burst-forming unit erythroid (BFU-E) and colony-forming unit—granulocyte; erythroid; macrophage and monocyte (CFU-GEMM) were counted on Days 5, 8 and 11 by a microscope according to the manufacturer’s protocol.

Intracellular ROS analysis

BM cells were incubated with biotin-conjugated lineage antibodies specific for murine CD5, Ter119, CD11b, CD45R/B220, and Gr-1 and stained with streptavidin-APC-Cy7, Sca1-PE-Cy7 and c-kit-APC. Then the cells were incubated with 10 μM DCFDA (St Louis, MO, USA) for 20 min at 37°C. The intracellular ROS levels in hematopoietic cells were analyzed by measuring the mean fluorescence intensity (MFI) of DCF by flow cytometry. For each sample, a minimum of 10 000 Lin+ cells was acquired (Fig. 1A).
To determine the protective effects of DTE on TBI-induced lethality in mice, we first observed the survival rates of mice after 7.0 and 7.5 Gy of TBI. As shown in Fig. 2A, TBI resulted in mortality in 83% of the vehicle-treated mice by Day 30 after 7.0 Gy TBI. In the irradiated mice treated with 50 mg/kg DTE, 100 mg/kg DTE, or 200 mg/kg DTE, mortality was observed in 54%, 47% and 17% of mice, respectively. Analysis of H2AX phosphorylation and NOX4 expression

After the BM cells were stained with the LSK antibodies as described above, the cells were fixed and permeabilized with BD Cytofix/Cytoperm buffer according to the manufacturer’s protocol. The cells were then stained with antibodies against H2AX phosphorylation or NOX4 and FITC-conjugated secondary antibodies. The H2AX phosphorylation and expression of NOX4 in the hematopoietic cells was determined by analyzing the MFI of FITC by flow cytometry.

Detection of SOD, MDA, CAT and GSHPx

The livers were homogenized in a saline solution. The activities of SOD (U/mg protein), MDA (nmol/mg protein), catalase (U/mg protein) and GSH (μmol/mg protein) were determined spectrophotometrically using their corresponding diagnostic reagent kits according to the manufacturer’s instructions. Values were determined by the colorimetric method. Protein content was determined by Bradford’s method using bovine serum albumin as a standard.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The analysis of variance (ANOVA) test was used to analyze differences between groups, and the t-test was used to analyze the difference between two groups.

RESULTS

Thirty-day survival after exposure to 7.0 Gy total body irradiation

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the mice at Day 30. The 30-day survival rates after TBI were 46%, 53% and 83% treated with 50, 100 and 200 mg/kg DTE. As shown in Fig. 2B, TBI resulted in mortality in 100% of the vehicle-treated mice by Day 30 after 7.5 Gy TBI. In the irradiated mice treated with 50 mg/kg DTE, 100 mg/kg DTE or 200 mg/kg DTE, the median survival days were 12, 12 and 20, compared with the 10 in the irradiation group. All the survival periods were significantly different from those of the vehicle-treated irradiated mice (P < 0.05). These results suggest that DTE effectively mitigates the TBI-induced lethality in mice.

DTE elevated peripheral blood cell counts after exposure to 6.0 Gy TBI

To determine whether the reduced TBI-induced lethality from DTE was due to increased hematopoiesis, we analyzed peripheral blood cell and bone marrow mononucleated cell (BMNCs) counts 9 days after the mice were exposed to 6 Gy TBI (Fig. 3). Compared with the control mice (7.5 ± 2.0) × 10^9 l^-1, the number of white blood cells (WBCs) in the ionizing radiation (IR) group (0.3 ± 0.0) × 10^9 l^-1 was significantly decreased (P < 0.01). The treatment with DTE elevated the WBC count (4.6 ± 1.5) × 10^9 l^-1, P < 0.05. In addition, the numbers of BMNCs from each femur were also determined (Fig. 3B). The number of BMNCs (3.0 ± 1.0) × 10^6 was significantly reduced in the vehicle-treated irradiated mice compared with in the non-irradiated control group (27.7 ± 2.6) × 10^6, P < 0.01. The number of BMNCs (11.3 ± 6.7) × 10^6 was increased in the DTE-treated irradiated mice compared with in the vehicle-treated mice, indicating that the DTE treatment promoted hematopoiesis after TBI.

The effect of DTE on hematopoietic colony-forming counts after exposure to 6.0 Gy TBI

To determine whether DTE increased hematopoiesis after TBI exposure by stimulating hematopoietic progenitor cells, we examined the effect of DTE on the CFU-GM frequency. As shown in Fig. 4, the data revealed that hematopoietic colony-forming counts (CFU-GM: 5.3 ± 1.5, BFU-E: 4.2 ± 1.5 and CFU-GEMM: 4.0 ± 1.0) in each 2 × 10^6 BMNCs of the IR group were markedly lower than those in the control group (12.8 ± 5.7, 11.8 ± 6.7, 17.2 ± 7.2, Fig. 3). After treatment with DTE, the hematopoietic colony-forming counts (6.7 ± 2.5, 8.8 ± 1.9, 10.8 ± 1.0) were partly restored.

The effects of DTE on hematopoietic DNA damage after exposure to 6.0 Gy TBI

We examined whether DTE treatment could reduce TBI-induced DNA damage by histone H2AX phosphorylation analysis, which has been widely used as a marker for DNA double-strand breaks (DSBs). As shown in Fig. 5, there was an increase in H2AX phosphorylation in BMNCs (24.8 ± 5.6) × 10^3, HPCs (25.8 ± 5.3) × 10^3 and HSCs (31.2 ± 6.1) × 10^3 of the IR group compared with in the control group (7.1 ± 2.3) × 10^3, (6.1 ± 2.1) × 10^3 and (11.1 ± 2.1) × 10^3, respectively. DTE treatment decreased H2AX phosphorylation in all three hematopoietic cells (BMNCs: (111.6 ± 2.3) × 10^3, HPCs: (14.2 ± 3.9) × 10^3, and HSCs: (20.0 ± 3.5) × 10^3) compared with in the irradiated mice (P < 0.05).

The effects of DTE on reactive oxygen species after exposure to 6.0 Gy TBI

To identify the mechanism underlying the effect of DTE, ROS levels were determined in the hematopoietic cells. As shown in Fig. 6, there was an increase in ROS levels in BMNCs (17.1 ± 3.1) × 10^3, HPCs (31.6 ± 7.9) × 10^3 and HSCs (32.0 ± 3.6) × 10^3 in the IR group compared with in the control group (6.4 ± 0.4) × 10^3, (13.2 ± 0.8) × 10^3 and (9.6 ± 2.0) × 10^3, respectively. The DTE treatment decreased the ROS levels in all three hematopoietic cells (BMNCs: (10.0 ± 1.0) × 10^3, HPCs: (10.7 ± 0.6) × 10^3 and HSCs: (14.1 ± 3.5) × 10^3) after exposure of the mice to 6.0 Gy TBI (P < 0.01).
The effects of DTE on the expression of NOX4 after exposure to 6.0 Gy TBI
We examined the expression of NOX4 in hematopoietic cells, which plays an important role in ROS production in the hematopoietic system. As shown in Fig. 7, an increase in NOX4 expression was detected in BMNCs (17.9 ± 5.7) × 10³, HPCs (16.7 ± 6.3) × 10³ and HSCs (21.6 ± 8.6) × 10³ in the IR group compared with in the control group (7.4 ± 3.3) × 10³, (6.2 ± 2.0) × 10³ and (9.8 ± 4.5) × 10³, respectively. DTE treatment decreased the expression of NOX4 (BMNCs (10.6 ± 4.4) × 10³, HPCs (9.3 ± 3.0) × 10³ and HSCs (10.2 ± 3.1) × 10³) after exposure to 6.0 Gy TBI (P < 0.05).

The effects of DTE on ROS after exposure to 6.0 Gy TBI
MDA has been used as a marker for lipid oxidation in the tissues, and 6.0 Gy irradiation significantly increased the level of lipid oxidation in the liver (Table 1). The DTE suppressed the irradiation-induced lipid oxidation (P < 0.05). The CAT, GSHPx and SOD were used to evaluate the anti-oxidant capacity. Irradiation of 6.0 Gy decreased the CAT, GSHPx and SOD in the liver (Table 1). DTE restored the irradiation-induced suppression of the CAT, GSHPx and SOD (P < 0.05).

DISCUSSION
The hematopoietic system is sensitive to IR. Acute radiation syndromes are often associated with infection, bleeding, and anemia from hematopoietic radiation injury. Development of an effective method to mitigate IR-induced hematopoietic injury remains an important research area for cancer radiotherapy and preparation for the possibility of nuclear accident and terrorism [18]. Previous studies have found that Chinese herbal medicine or extracts may be able to mitigate TBI-induced injuries in the brain, esophagus, hematopoietic system and intestine of irradiated animals [19–26]. In the present study, we investigated whether DTE had protective effects against hematopoietic radiation injury.
In this study, the effects of DTE on the survival rates of mice were evaluated first. It was found that 50 mg/kg DTE, 100 mg/kg DTE and 200 mg/kg DTE all significantly elevated the survival rate of mice exposed to 7.0 and 7.5 Gy TBI, which indicated that DTE could protect mice from IR. Protective effects of DTE on WBCs, BMNC and CFU-GM potential in mice exposed to 6.0Gy TBI were confirmed [27, 28]. These data suggested that DTE might alleviate TBI-induced acute radiation syndromes by increasing the differentiation ability of bone marrow cells.

Radiation acts on the body, then, through direct effects on the water content of the cells, producing a large number of ROS and damaging the body's redox balance [29]. Rays can also directly act

**Table 1. Effects of DTE on the CAT, GSHPx, MDA and SOD in the liver after 6.0 Gy TBI**

| Group          | CAT (U/mg protein) | GSHPx (μmol/mg protein) | MDA (nmol/mg protein) | SOD (U/mg protein) |
|----------------|--------------------|-------------------------|-----------------------|--------------------|
| Control        | 18.13 ± 2.28       | 454.90 ± 33.70          | 5.72 ± 0.10           | 477.25 ± 35.58     |
| 6.0 Gy         | 9.29 ± 3.07        | 252.30 ± 41.93          | 8.06 ± 0.27           | 207.00 ± 21.00     |
| 6.0 Gy + DTE   | 13.61 ± 3.78*      | 361.30 ± 66.01*         | 6.07 ± 0.02*          | 322.58 ± 47.00*    |

*Value are means ± SD, n = 5. Before and after the mice were exposed to 6 Gy TBI, they were administrated with vehicle (6 Gy) and DTE (200 mg/kg) as described in the Methods. The liver were homogenized and detected by their corresponding diagnostic reagent kits according to the manufacture's instructions.* Compared with 6 Gy group, \( P < 0.05. \)
on the biological macromolecules in cells, destroying DNA and also the expression of proteins in cells [30], activating a variety of signaling molecules and signaling pathways in cells, resulting in cell apoptosis, cancers and even death of the organism [31,32]. Previous reports have shown the antioxidant ability of tea [13,14]. In this study, the ROS levels in hematopoietic cells were determined. Our results confirmed that DTE could decrease the ROS levels elevated by IR. In addition, DNA injury induced by IR was determined by H2AX phosphorylation, and this was used as an index for quantifying DNA double-strand breaks [33–36]. The data suggested that DTE could mitigate DNA injury induced by IR.

ROS levels in the cells were determined by ROS production and degradation [37]. Previous studies have shown that NOX4 and MDA play important roles in ROS production, and that CAT, GSHPx and SOD are important antioxidant enzymes [27,38–40]. Thus, the expression level of NOX4 in hematopoietic cells was determined (by flow cytometry). Our results showed that DTE can effectively decrease NOX4 expression in hematopoietic cells. In addition, the MDA, CAT, GSHPx and SOD in the liver were determined, and our data showed that DTE could partly restore the CAT, GSHPx and SOD.

CONCLUSION
Our studies show protective effects of DTE against radiation-induced injury. However, it is difficult to determine which components are effective. Thus, more detailed research is needed.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests

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