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Original Article

Combination treatment of cordycepin and radiation induces MA-10 mouse Leydig tumor cell death via ROS accumulation and DNA damage

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Abstract: Leydig cell tumor is the most frequent non-germ cell tumors of testis. The biggest challenge of using radiotherapy to treat testicular cancer is in effectively killing cancer cells and maintaining reproductive function after treatment. Our recently published article showed that cordycepin could enhance radiosensitivity to induce mouse Leydig tumor cell apoptosis by inducing cell cycle arrest, caspase pathway and endoplasmic reticulum (ER) stress. In the present study, the potency and mechanism of a previous combination treatment protocol on reactive oxygen species (ROS) induction and DNA damage were further investigated. Our results reveal that 25 μM cordycepin plus 4 Gy radiation leads to ROS accumulation accompanied by a decrease in heme oxygenase (HO)-1 protein expression in MA-10 mouse Leydig tumor cells. Subsequently, pronounced DNA damage with phosphorylated H2A histone family member X (γ-H2AX) increase and activation of DNA damage-related signaling pathways including double and single stranded break-induced ataxia telangiectasia mutated (ATM)/checkpoint kinase (Chk)2 and ataxia telangiectasia mutated and Rad3 related (ATR)/Chk1 signaling axes were identified. p53-dependent pathway was then initiated ultimately leading to cell death. Preincubated with free radical scavenger, N-acetylcysteine (NAC), down-regulated γ-H2AX expression in treated cells and partially reduced cell death, indicating that ROS overproduction is involved in combination treatment-induced DNA damage. Furthermore, the combination treatment effectively inhibited tumor growth as reflected in the reduction of tumor volume, size and weight, and high expression level of γ-H2AX in tumor tissue *in vivo*, suggesting that the combination treatment inhibited tumor growth via causing DNA damage in MA-10 cells. In summary, these results expound that the combination treatment of cordycepin and radiation induces MA-10 mouse Leydig tumor cell death through ROS accumulation and DNA damage. This finding can serve as a reference guideline for future clinical therapy of testicular cancer and provide potential targets for anti-cancer drug design.

Keywords: Cordycepin, radiation, Leydig cell tumor, MA-10 cell, ROS, DNA damage, HO-1, ATM, ATR, γ-H2AX

Introduction

Primary testicular neoplasms include two predominant groups, germ cell tumors and sex cord stromal tumors [1]. Leydig cell tumor is a sex cord stromal tumor that arise from Leydig cells which comprise 1% to 3% of all testicular neoplasms [2]. Although it can occur at any age, prepubertal boys between 6 and 10 years old and men between 20 and 60 years old are the predominant ages for Leydig cell tumor [3]. At present, the gold standard for clinical treatment of malignant Leydig cell tumor is radical orchiectomy, but patients will completely lose their fertility after receiving the treatment [4].

Radiotherapy is a localized cancer treatment method that uses high-energy particles such as x-rays, gamma rays or electron beams to kill cancer cells. It can cause cancer cell death via

direct and indirect effects [5]. In direct effect, radiation can induce single stranded breaks and double stranded breaks in DNA and lead to cancer cell apoptosis [6]. In the case of indirect effect, radiation can activate radiolysis of water to generate reactive oxygen species (ROS). The ROS accumulation then induces cellular stress and ultimately alter cellular signaling pathways [7]. However, testis is one of the radiation sensitive tissues, and dose of radiation above 4 Gy could lead to azoospermia in most men [8]. Moreover, Leydig cells are significantly more radiation resistant than other germ cells, so higher radiation dose is required to kill Leydig cell tumors [9]. Although effective cancer treatment is crucial, the side effects of gonadal damage can cause considerable distress to patients, especially in those of reproductive age. Therefore, looking for a substance that can increase the radiosensitivity of Leydig cell tumors, unravel its molecular mechanism, and applying it in combination therapy to reduce the radiation dose used in the treatment are important.

Cordycepin is the main functional component of *Cordyceps sinensis* which has been used extensively as an herbal complementary and alternative medicine drug to treat different illnesses based on its multiple pharmacological activities such as immune modulation, hormone regulation, apoptosis induction, and tumor growth inhibition [10-12]. It has been reported that cordycepin could elevate intracellular ROS levels to eliminate human tongue cancer cells and mouse Leydig tumor cells [13, 14]. In addition, cordycepin treatment could sensitize breast carcinoma cells toward irradiation through modulation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase (HO)-1/ROS signaling axis to increase ROS accumulation [15]. The production of ROS might induce endoplasmic reticulum (ER) stress, which eventually leads to apoptosis [16]. HO-1 protein is recognized as a critical oxidative stress sensor and regulatory molecule [17]. Previous studies demonstrated that HO-1 protein is increased in several cancers and plays a vital role in defending cancer cells from excessive ROS [18]. Down-regulation of HO-1 protein expression might cause ROS accumulation in cancer cells.

The significant endogenous DNA damage is initial from chemical activation, for instance, ROS activation [19]. Exogenous DNA damage usually occurs when physical agents such as ionizing radiation damage the DNA. Ionizing radiation can directly cause DNA double stranded breaks or damage the DNA by radiolysis of the surrounding water to generate reactive hydroxyl radicals [20]. Phosphorylated H2A histone family member X (γ-H2AX), a hallmark of DNA damage, play an important role in regulating repair activities and targeting to DNA double stranded break sites. Ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia mutated and Rad3 related (ATR) are two DNA-damage-sensing proteins belong to the phosphoinositide 3-kinase (PI3K) family [21]. These two proteins are similar in structure and function, which can be activated by double stranded and single stranded DNA break, respectively. A relevant substrate of ATM and ATR are checkpoint kinase (Chk)2 and Chk1 kinases which can be phosphorylated and activated following by ATM and ATR activation, respectively [22]. The tumor suppressor p53 is a central player in a network that protects eukaryotic cells from damage, which might act as a node for organizing whether the cell responds to different types and levels of damage with DNA repair, cell cycle arrest, apoptosis, or autophagy [23, 24]. p53 could be phosphorylated and activated by several kinases in response to DNA damage, including ATR and Chk1 as well as ATM and Chk2, resulting in a cross-talking between two pathways [24, 25]. Activation of p53 also leads to the transcription of pro-apoptotic genes, including Bcl-2-associated X protein gene (BAX), p53 upregulated modulator of apoptosis gene (Puma), and apoptotic protease activating factor 1 gene (Apaf1), leading to apoptosis if the damage persists [26].

Recently, we have reported that cordycepin could enhance radiosensitivity to induce MA-10 mouse Leydig tumor cell apoptosis through cell cycle arrest, caspase pathway and ER stress [27]. However, whether the combination treatment with cordycepin and radiation-induced Leydig tumor cell death is related to the accumulation of intracellular ROS and the induction of DNA damage is still a mystery. In this study, the potency and mechanism of a previous combination treatment protocol on ROS induction and DNA damage were further examined. We found that 25 μM cordycepin plus 4 Gy radiation treatment induced ROS accumulation and accompanied by an inhibition of HO-1 protein

expression in MA-10 cells. DNA damage with γ-H2AX increase and activation of related signaling pathways including ATM/Chk2/p53 and ATR/Chk1/p53 were evident. In addition, MA-10 tumor-bearing mice receiving combination treatment with cordycepin plus radiation exhibited superior tumor growth inhibition and upregulated γ-H2AX protein expression in tumor tissue suggesting that the MA-10 Leydig tumor cells die from DNA damage. Taken together, these results reveal an important role for combination treatment of cordycepin and radiation in inducing Leydig cell death through ROS accumulation and DNA damage.

Materials and methods

Cell culture

MA-10 mouse Leydig tumor cell line was obtained from Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA). Cells were cultured in Waymouth's MB 752/1 medium containing 10% fetal bovine serum (FBS) with 1% penicillin and streptomycin, and 20 mM HEPES at 37°C in a humidified environment containing 5% CO_2. In experiments for testing the effect and mechanism of the treatments, the FBS content of the culture medium was reduced to 2% to prevent the complex protein composition in FBS from affecting the pharmacological effects.

Chemicals

Cordycepin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. DMSO was used to dissolve the cordycepin in final concentration (80 mM) of cordycepin solution for stock. The stock solution is then further diluted with cell culture medium to working concentrations for experimental use.

Irradiation treatment

Irradiation treatment was conducted by a linear accelerator with 6 MV X-rays at a dose rate of 5 Gy/min. An additional 2 cm of a tissue-equivalent bolus was placed on the top of the flask or mouse to ensure electronic equilibrium, and 10 cm of tissue-equivalent material was placed under the tissue culture flask or mouse to get full backscatter.

Determination of ROS production

At the desired time points, cells from different treatment groups were harvested by trypsin, washed, and then stained with 2 μM dichlorodihydrofluorescein diacetate (DCF-DA) in Waymouth's MB 752/1 medium at 37°C for 30 min. The stained cells were washed, resuspended in ice-cold PBS, and immediately analyzed using CytoFLEX flow cytometry with CytExpert software. In some experiments, the test cells were pre-incubated in the absence or presence of 2.5 mM N-acetylcysteine (NAC) for 1 hr before treatment. Cellular ROS levels were estimated by the methods described above.

Protein extraction and western blotting

Cells from different treatment groups were lysed by 100 μl lysis buffer with proteinase inhibitor for 30 min at room temperature. Cell lysates were centrifuged at 12,000 rpm for 12 min at 4°C. The supernatants were collected and stored at -20°C until future analysis. Before running western blotting, protein concentrations of cell lysates were measured by Bio-Rad protein assay dye reagent concentrate according to the manufacturer's instructions. For western Blotting, protein samples (25 μg/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) with standard running buffer at room temperature, and then electrophoretically transferred to polyvinyldifluoride (PVDF) membrane at 4°C. The membrane was blocked with 5% nonfat milk, washed by tris-buffered saline with 0.1% Tween 20 detergent (TBST), and subsequently incubated with various primary antibodies (Abs), HO-1 Abs (GeneTex, Cat#GTX101147, 1:1000 dilution), β-actin Abs (Sigma-Aldrich, Cat#A5441, 1:8000 dilution), γ-H2AX Abs (Abcam, Cat#ab124781, 1:1000 dilution), ATM Abs (Cell Signaling, Cat#2873, 1:1000 dilution), phosphorylated (p)-ATM (Ser1981) Abs (GeneTex, Cat#GTX30636, 1:1000 dilution), Chk2 Abs (Cell Signaling, Cat#2662, 1:1000 dilution), p-Chk2 (phosphor T68) Abs (Abcam, Cat#ab278548, 1:1000 dilution), ATR Abs (GeneTex, Cat#GTX128146, 1:1000 dilution), p-ATR (Ser428) Abs (Cell Signaling, Cat#2853, 1:1000 dilution), Chk1 Abs (Cell Signaling, Cat#2360, 1:1000 dilution), p-Chk1 (Ser317) Abs (Cell Signaling, Cat#12302, 1:1000 dilution), p53 Abs (Cell Signaling, Cat#25245, 1:1000 dilution) or p-p53 Abs (Cell Signaling, Cat#92845, 1:1000 dilution), overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (PerkinElmer, Cat#NEF82200-1EA, 1:2000 dilution) or HRP-conjugated anti-

rabbit IgG (PerkinElmer, Cat#NEF81200-1EA, 1:2000 dilution) Abs for 1 hr at room temperature. Bands were detected by enhanced chemiluminescence kit through UVP EC3 BioImaging System. The optical density of each protein band was quantitated by using ImageJ software. The amount of β-actin in each lane was detected as a control to correct the expression of various proteins.

MTT cell viability test

MA-10 cells were seeded in 96-well plate containing 6 × 10^3 cells with 100 μl cell culture medium in each well. After 70-80% confluence, cells were preincubated without or with 2.5 mM NAC for 1 hr and then treated without or with different concentrations of cordycepin and/or radiation for different durations, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added at different time points with the final concentration of 0.5 mg/ml, and then incubated at 37°C for 4 hr. After incubation, the medium was removed and 100 μl DMSO was added into each well to dissolve the crystals by shaking the plate weakly in dark. The O.D. values in each treatment were determined at λ = 570 nm by ELISA reader.

Comet assay

DNA damage was determined by CometAssay™ kit (4250-050-K, TREVIZEN, Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, 1 × 10^5 cells were collected in 50 ml ice-cold PBS. The suspended cells were then mixed with completely melted low-melting agarose (LMA). Cell/agarose mixture was spread on the comet slide and incubated at 4°C for 30 min to solidify the agarose. After congelation, the slides were incubated in lysis solution at 4°C for 60 min. Then, the slides were moved carefully into alkaline solution (pH>13) and incubated at room temperature for 30 min. For neutralization, the slides were washed once in 1 × Tris-borate-EDTA (TBE) buffer and electrophoresis was performed at 31 V for 40 min. The slides were then washed with 70% ethanol and air dried after removing excess liquid. Cell events were than stained with SYBR® Green dye and visualized under a fluorescence microscope. The signals of the head and tail DNA from 100 × magnification fields were analyzed to estimate DNA damage in each group. Percentage of tail DNA content and tail moment (tail length × % of DNA in the tail) values were measured by the "OpenComet" program of the ImageJ software.

Animal experiments

Male 4 to 5-week-old C57BL/6 mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Experimental procedures were consistent with ethical principles for animal research which were approved by the Institutional Animal Care and Use Committee (IACUC) of National Cheng Kung University (IACUC Approval No. 110095). Mice were subcutaneously inoculated with 1 × 10^6 MA-10 cells suspended in 0.1 ml Waymouth medium into the back. 7 days post inoculation, mice were randomly assigned to 4 groups including (A) Control group: mice were injected intraperitoneally (i.p.) with vehicle; (B) Cordycepin group: mice were i.p. injected with 20 mg cordycepin/kg thrice a week for 2 weeks; (C) Radiation group: mice were treated with a single dose of 4 Gy radiation; (D) Combination group: mice received the combination treatment of 20 mg cordycepin/kg thrice a week and a single dose of 4 Gy IR at the beginning of the first week. The size of tumor was measured with a clipper twice a day. Tumor volume was calculated by the formula: 0.52 × length × width × width. When mice were sacrificed, MA-10 tumor tissues were collected, weighted, and photographed. Tissue samples were then fixed by 4% paraformaldehyde solution and embedded by paraffin for immunohistochemistry.

Immunohistochemistry staining

MA-10 tumor tissue sections were dewaxed by xylene, dehydrated by ethanol, and blocked endogenous peroxidase activity by incubating with 0.3% H_2O_2. The sections were infiltrated in sodium citrate buffer (10 mM sodium citrate and 0.05% tween 20, pH 6.0) for 50 min at 120°C autoclave for antigen retrieval. Samples were then blocked with 2% nonfat milk for 1 hr and incubated with primary Ab for γ-H2AX (Abcam, Cat#ab124781, 1:500 dilution) detection at 4°C overnight. Signals were visualized using HRP-conjugated goat anti-rabbit IgG secondary Ab (PerkinElmer, Cat#NEF81200-1EA, 1:2000 dilution) and the chromogenic substrate 3,3-diaminobenzidine (DAB). The sections were then counterstained with hematoxy-

lin and mounted under coverslips. Sample slides were examined with a light microscope. DAB positive area was analyzed by the "area detection" program of the ImageJ software and the percentage of positive area was calculated.

Statistical analysis

The data are expressed as mean ± SEM of three separate experiments. Statistical significance of difference was determined by One-way or Two-way ANOVA with Tukey's test for multiple comparison. The statistical significance was considered as $P<0.05$ in all experiments.

Results

Combination treatment led to ROS accumulation and accompanied by suppression of HO-1 protein expression in MA-10 cells

Recently, we have published that cordycepin could enhance radiosensitivity to induce mouse Leydig tumor cells toward apoptosis via cell cycle arrest, caspase pathway and ER stress [27]. In the present study, the effective dose of the combination treatment used in previous study, 25 μM cordycepin plus 4 Gy radiation, was utilized to further explore the molecular mechanism by which the combination treatment acts on Leydig tumor cells. The effect of the combination treatment on intracellular ROS accumulation in MA-10 mouse Leydig tumor cell was first analyzed with DCF-DA-based method. Upregulation of ROS generation could be detected with an increase of DCF-DA intensity. The results showed that an approximately 2-fold increase of DCF-DA mean fluorescence intensity (MFI) at 6 hr post MA-10 cells treating with 4 Gy radiation alone or the combination of 25 μM cordycepin plus 4 Gy radiation but not 25 μM cordycepin alone were detected (**Figure 1A** and **1B**). Obviously, the ROS level significantly increased 3.7-fold in MA-10 cells treated with the combination of 25 μM cordycepin plus 4 Gy radiation at 24 hr comparted to the untreated control group ($P<0.05$) (**Figure 1A** and **1B**). At the same time point, the DCF-DA MFI of MA-10 cells treated with cordycepin alone and radiation alone represented 3-fold and 1.6-fold increases, respectively (**Figure 1A** and **1B**). These data indicated that the combination treatment was able to induce ROS generation and displayed a better ROS-inducing ability than the treatment with cordycepin alone or radiation alone in MA-10 cells. Next, we further analyzed the expression profile of HO-1 protein, a critical oxidative stress sensor and regulator, by western blotting. The results demonstrated that the HO-1 protein expression levels were reduced in MA-10 cells treated with cordycepin alone and the combination of cordycepin plus radiation at 6 hr and 24 hr (**Figure 1C** and **1D**). The combination treatment showed the strongest negative regulatory effect on the expression of HO-1 protein in MA-10 cells. Visibly, the expression of HO-1 protein was significantly reduced by approximately 50% at 24 hr post treatment ($P<0.05$) (**Figure 1C** and **1D**). These evidences suggest that the combination treatment effectively induced ROS accumulation and accompanied by HO-1 protein downregulation in MA-10 mouse Leydig tumor cells.

Combination treatment induced DNA damage in MA-10 cells

Since ROS can drive DNA damage and affect the DNA damage response [28], we next need to clarify whether the combination treatment of cordycepin and radiation can induce DNA damage in MA-10 cells. To address this issue, we used Comet assay to analyze the extent of DNA damage in MA-10 cells treated with 25 μM cordycepin alone, 4 Gy radiation alone or in combination. Data demonstrated that most of cell events in both single and combination treatment groups containing damaged DNA respecting by the appearance of a comet with a bright head and tail (**Figure 2A**). In contrast, undamaged DNA displayed in the most cell events of control group as an intact nucleus with no tail (**Figure 2A**). Interestingly, the representative cellular event of combination treatment group displayed longer and larger comets compared to the cordycepin alone and radiation alone groups, indicating that larger amount of DNA breaks happened in MA-10 cell treated with cordycepin plus radiation (Bottom left corner of each image in **Figure 2A**). In the results of quantitative analysis, the percentage of tail DNA in all treatment groups (cordycepin alone: 15.1%; radiation alone: 15.6%; combination: 17.2%) were significantly higher than that in the control group (6.5%), indicating that both single and combination treatments can effectively cause DNA damage in MA-10 cells (**Figure 2B**).

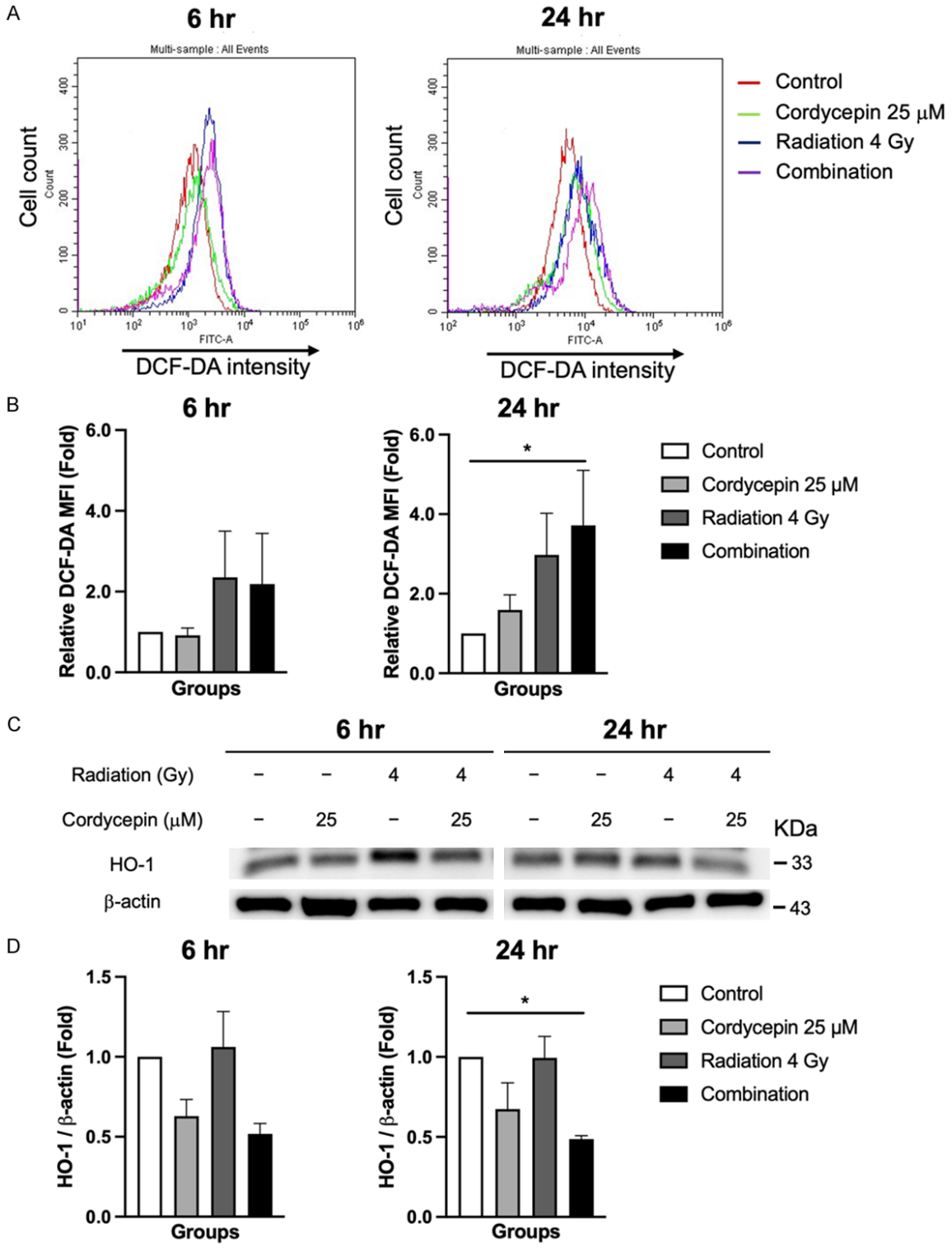

Figure 1. Combination treatment of cordycepin and radiation induced ROS accumulation and down-regulated HO-1 protein expression in MA-10 cells. MA-10 cells were treated with vehicle, 25 μM cordycepin, 4 Gy radiation, or the combination of 25 μM cordycepin plus 4 Gy radiation for 6 and 24 hr, respectively. Cells were stained with DCF-DA for 30 min and the fluorescence intensity was detected by flow cytometry (A). Mean fluorescence intensities (MFI) were quantitated and data were presented as mean ± SEM of three independent experiments (B). The protein expression levels of HO-1 and β-actin were detected by western blotting (C). The integrated optical densities of HO-1 protein were normalized with corresponding β-actin protein and data were presented as mean ± SEM of three independent experiments in fold change relative to the control group (D). $^{*}P<0.05$ represents significantly statistical difference compared to the control group.

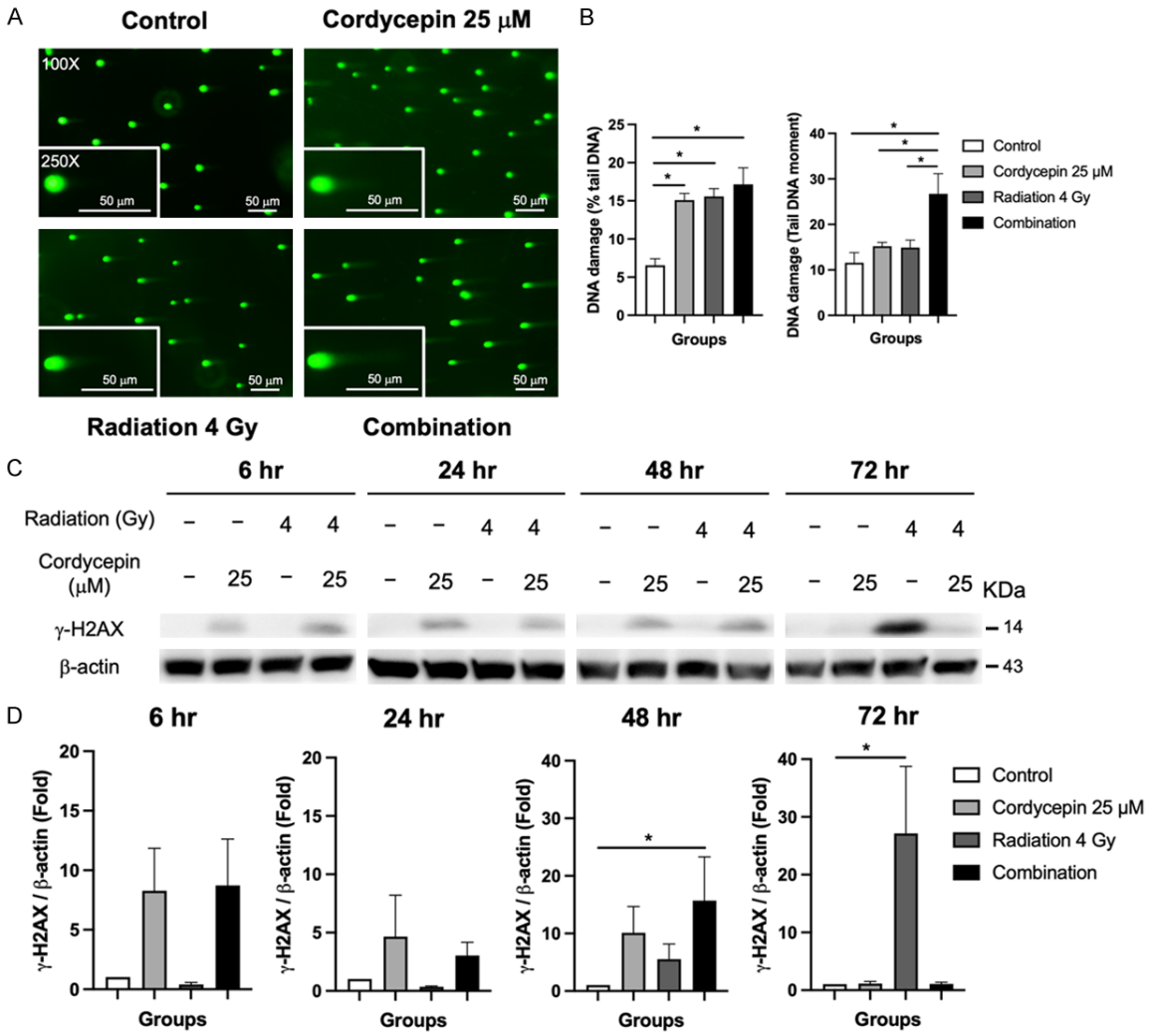

Figure 2. Combination treatment of cordycepin and radiation induced DNA damage and up-regulated γ-H2AX protein expression in MA-10 cells. MA-10 cells were treated with vehicle, 25 μM cordycepin, 4 Gy radiation, or the combination of 25 μM cordycepin plus 4 Gy radiation for 6, 24, 48 and 72 hr, respectively. Comet assay was performed to measure the DNA comet tail at 24 hr post treatment. Images of cells analyzed by comet assay were photographed under a fluorescence microscope at 100 × magnification and 250 × digital magnification (A). Percentage of tail DNA content and tail moment (tail length × % of DNA in the tail) were analyzed by OpenComet program of ImageJ software and data were presented as mean ± SEM of independent cell events in the image field (B). The protein expression levels of γ-H2AX and β-actin were detected by western blotting (C). The integrated optical densities of γ-H2AX protein were normalized with corresponding β-actin protein and data were presented as mean ± SEM of three independent experiments in fold change relative to the control group (D). $^{*}P<0.05$ represents significantly statistical difference compared to every other group (B) or the control group (D).

In addition, the tail DNA moment value of combination treatment group (26.8) was significantly higher than that of the cordycepin alone (15.2), radiation alone (15.0) and the control (11.6) groups, reflecting that the combination treatment has the best DNA damage induction ability (**Figure 2B**). When DNA damage happen, γ-H2AX is the first step in recruiting and localizing DNA repair proteins [29]. We next analyzed the expression of γ-H2AX at different time points post treatment. The western blotting results showed that both the cordycepin alone and the combination treatment group could be detected an increase in the expression of γ-H2AX at 6 hr, 24 hr, and 48 hr (**Figure 2C** and **2D**). The γ-H2AX expression in the combination treatment group was significantly higher than that in the control group at 48 hr ($P<0.05$) (**Figure 2C** and **2D**). However, the expression of γ-H2AX could not be significantly elevated until

72 hours after MA-10 cells treating with radiation alone (**Figure 2C** and **2D**). Taken together, the combination treatment has the best ability to induce DNA damage in MA-10 cells, and the addition of cordycepin may help radiation induce an early expression of γ-H2AX in advance to facilitate the occurrence of DNA damage.

Combination treatment activated DNA damage-related signaling pathways

Studies have shown that double stranded and single stranded DNA break can activate ATM/Chk2 and ATR/Chk1 signaling axes, respectively, and finally initiate the p53-dependent pathway to induce apoptosis if the DNA damage persists [24]. To determine whether the DNA damage caused by combination treatment of cordycepin and radiation could further activate DNA damage-related signaling pathways, the total and phosphorylated ATM, Chk2, ATR, Chk1 and p53 were analyzed by western blotting. The results showed that the expression of the phosphorylated ATM protein in MA-10 cells under the untreated control was very low, and slightly increased by treating with cordycepin alone, radiation alone, or in combination between 24 hr to 48 hr (**Figure 3A** and **3B**). Markedly, the combination treatment significantly increased the ratio of phosphorylated Chk2 protein per total Chk2 protein expression levels with 2.1-fold and 4.5-fold at 24 hr and 48 hr, respectively, compared to the control group ($P<0.05$) (**Figure 3C** and **3D**), reflecting that the ATM/Chk2 signaling axis was activated by DNA double-stranded break in cordycepin plus radiation-treated MA-10 cells. On the other hand, the expression levers of phosphorylated ATR protein were slightly enhanced in MA-10 cells treated with cordycepin alone, radiation alone, or in combination at 24 hr and 48 hr (**Figure 3E** and **3F**). Obviously, the ratios of phosphorylated Chk1 protein per total Chk1 protein expression levels were significantly elevated by cordycepin alone and the combination treatment with 3.1-fold and 2.9-fold in 24 hr, respectively ($P<0.05$), and the combination treatment group sustained this level of response up to 48 hr (**Figure 3G** and **3H**). The results indicated that the ATR/Chk1 signaling axis was activated by DNA single-stranded break in cordycepin plus radiation-treated MA-10 cells. Furthermore, the ratios of phosphorylated p53 protein per total p53 protein expression levels were significantly increased in MA-10 cells treated with the cordycepin plus radiation in 24 hr (5.0-fold) and 48 hr (2.5-fold) compared to the control group ($P<0.05$), indicating that combination treatment can effectively activate the p53-dependent signaling pathways (**Figure 3I** and **3J**). These data suggest that the combination treatment-induced DNA damage further activated DNA damage-related signaling pathways in MA-10 cells.

DNA damage and viability reduction of MA-10 cells induced by combination treatment are partially due to the accumulation of ROS

To verify whether the DNA damage induced by combination treatment is due to the overproduction of ROS, MA-10 cells were preincubated with a ROS scavenger, NAC, for 1 hr and then treated with vehicle or 25 μM cordycepin plus 4 Gy radiation for additional 24 hr. Intracellular ROS levels were determined by DCF-DA-based method using flow cytometry. The results demonstrated that the combination treatment significantly increased ROS levels, but preincubation with NAC fully reversed this effect as showing by the DCF-DA intensity peaks on the flow cytometry histogram (**Figure 4A**). Quantitative analysis further indicated that combination treatment induced 1.7-fold increase on relative DCF-DA MFI compared to the control group, but ROS level was totally restored in the presence of NAC ($P<0.05$) (**Figure 4B**). Interestingly, the expression levels of γ-H2AX of cordycepin alone, radiation alone, or combination treatment group were partially restored in the presence of NAC reflecting the phenomenon of DNA damage in MA-10 cells partially contributed by ROS accumulation ($P<0.05$) (**Figure 4C** and **4D**). Moreover, the cell viability was significantly reduced after MA-10 cells treating with cordycepin plus radiation (Control v.s. Combination: 100% v.s. 18%; $P<0.05$), but the level was partially restored in the presence of NAC (Combination v.s. Combination + NAC: 18% v.s. 40%; $P<0.05$) (**Figure 4E**). These results suggest that DNA damage and viability reduction of MA-10 cells induced by the combination treatment are partially due to the accumulation of ROS.

Combination treatment inhibit MA-10 tumor growth in mice

Based on the superior ROS-inducing and DNA-damage-causing capabilities of the combina-

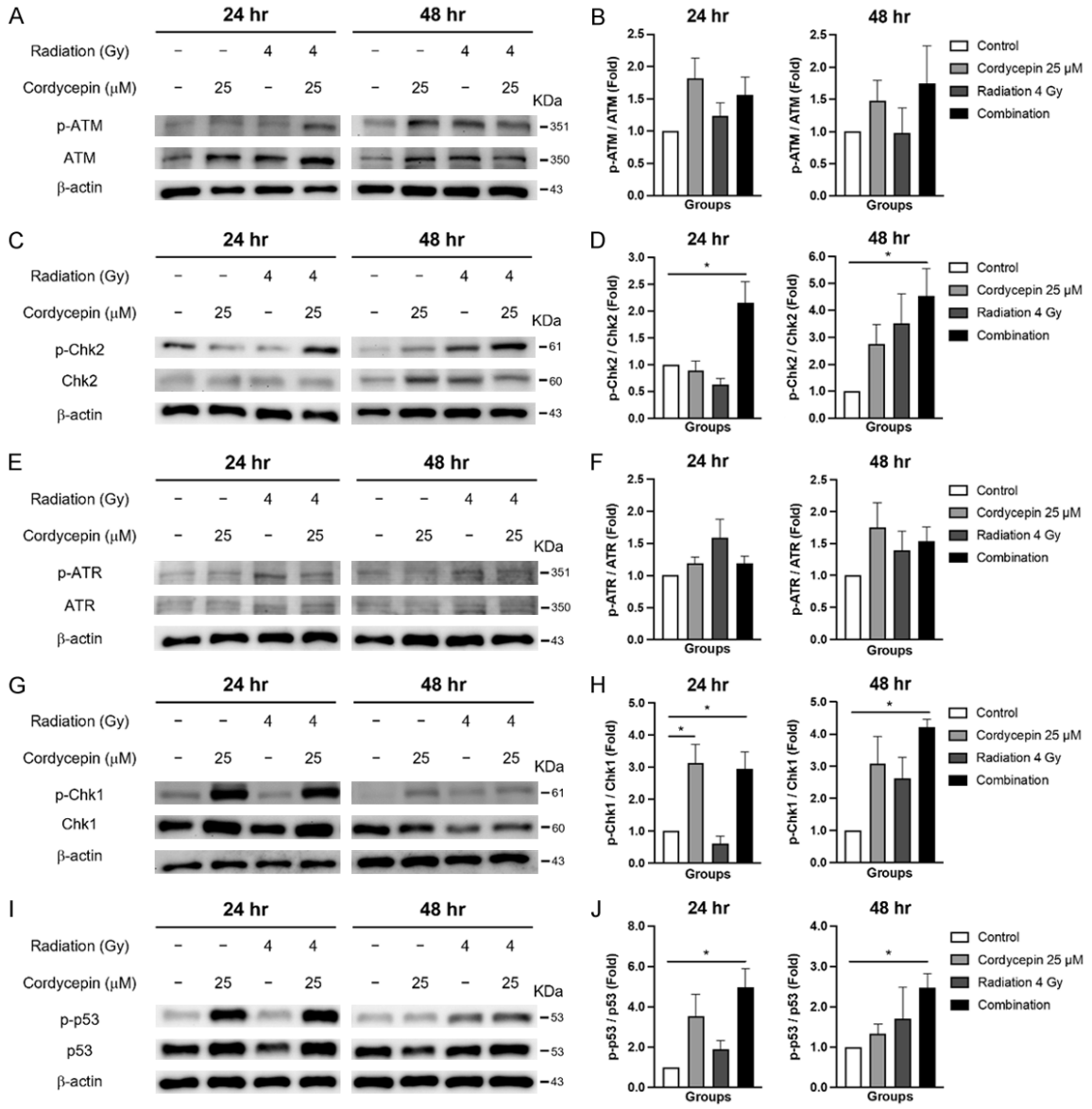

Figure 3. Combination treatment of cordycepin and radiation activated DNA damage-related signaling pathways in MA-10 cells. MA-10 cells were treated with vehicle, 25 μM cordycepin, 4 Gy radiation, or the combination of 25 μM cordycepin plus 4 Gy radiation for 24 and 48 hr, respectively. The protein expression levels of total and phosphorylated ATM (ATM and p-ATM), Chk2 (Chk2 and p-Chk2), ATR (ATR and p-ATR), Chk1 (Chk1 and p-Chk1), and p53 (p53 and p-p53) and β-actin were detected by western blotting (A, C, E, G, I). The integrated optical densities of p-ATM (B), p-Chk2 (D), p-ATR (F), p-Chk2 (H), and p-p53 (J) proteins were determined after total ATM, Chk2, ATR, Chk1, and p53 proteins normalization, respectively. Results in (B, D, F, H and J) were presented as mean ± SEM of three independent experiments. $^{*}P<0.05$ represents significantly statistical difference compared to the control group.

tion treatment *in vitro*, we further used a C57BL/6 mouse model to examine the treated mice for an *in vivo* protective effect against challenge by MA-10 mouse Leydig tumor cells. C57BL/6 mice were treated with cordycepin alone, radiation alone, or in combination staring from 8th days after inoculation with MA-10 cells. The results showed that the transplanted MA-10 tumor volumes of the mice receiving the intraperitoneal injection of 20 mg/kg cordycepin three times a week for two weeks were slightly reduced with statistical difference compared to the control group and other treatment groups (**Figure 5A**). Moreover, the transplanted MA-10 tumor volumes of mice received a single dose of 4 Gy radiation treatment were moder-

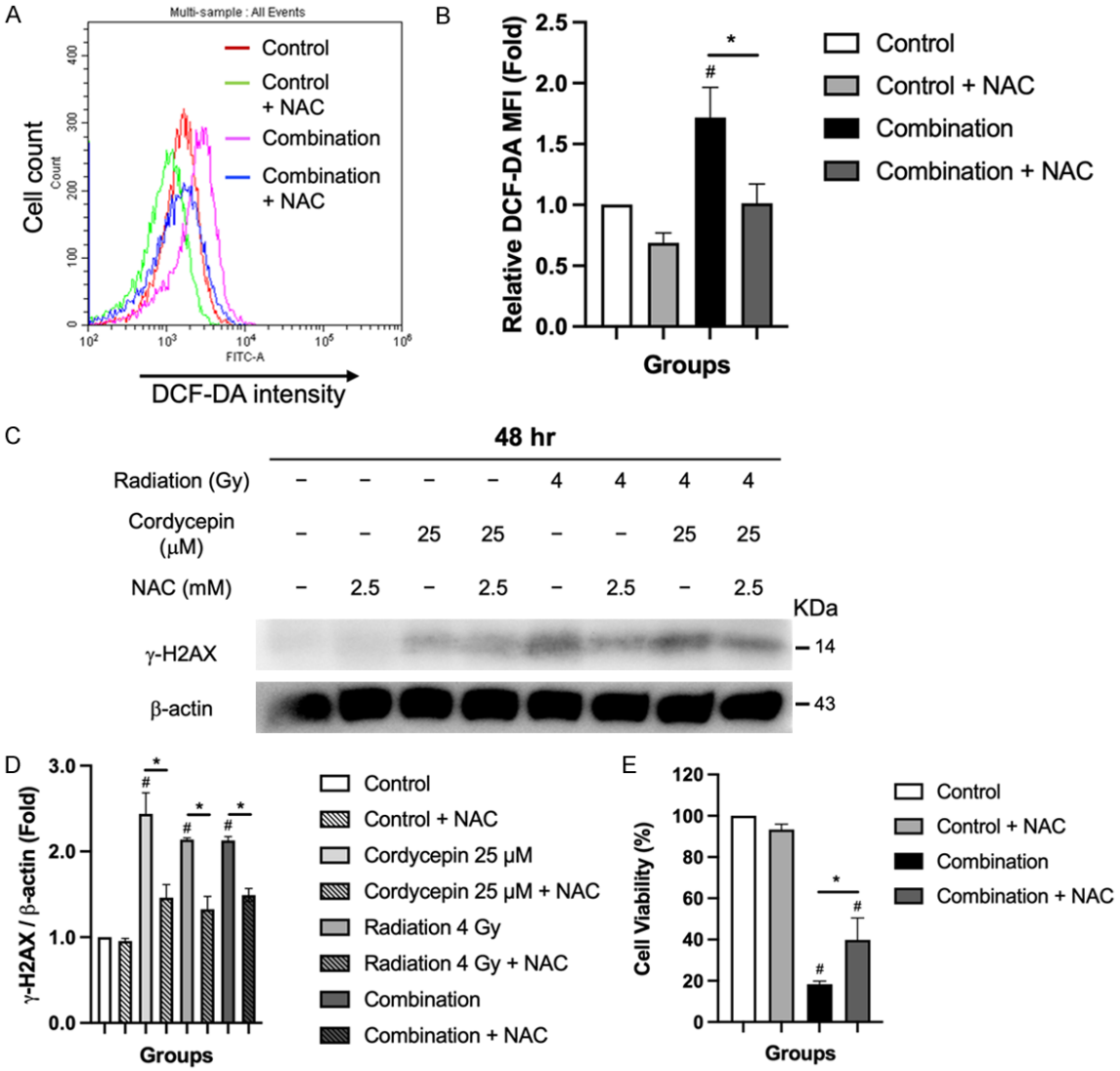

Figure 4. N-acetyl-L-cysteine (NAC) administration reduced combination treatment-induced ROS generation and γ-H2AX expression, and reversed cell viability in MA-10 cells. MA-10 cells were pretreated without or with 2.5 mM NAC for 1 hr and then cotreated without or with the combination of 25 μM cordycepin plus 4 Gy radiation for 24 hr or 48 hr, respectively. Cells were stained with DCF-DA for 5 min before NAC treatment and the fluorescence intensity was detected by flow cytometry (A). Mean fluorescence intensities (MFI) were quantitated and data were presented as mean ± SEM of three independent experiments (B). The protein expression levels of γ-H2AX and β-actin at 48 hr post treatment were detected by western blotting (C). The integrated optical densities of γ-H2AX protein were normalized with corresponding β-actin protein and data were presented as mean ± SEM of three independent experiments in fold change relative to the control group (D). Cell viability was measured by MTT assay and results are presented as mean ± SEM of three independent experiments in percentages of cell viability relative to the control group (E). $^{*}P<0.05$ represents significantly statistical difference between NAC-untreated and -treated groups. $^{\#}P<0.05$ represents significantly statistical difference compared to the control group.

ately reduced with statistical difference compared with the control group and other treatment groups (**Figure 5A**). Strikingly, mice treated with cordycepin plus radiation displayed the most potent inhibitory effect on tumor volume in between all groups ($P<0.05$) reflecting that the combination treatment synergistically inhibited tumorigenesis of MA-10 cells in C57BL/6 mice (**Figure 5A**). During evaluation of the antitumor activity period, there was no significant difference in body weight changes between mice receiving different treatments and mice in the control group, indicating that the treatment intervention did not cause toxicity to the mice

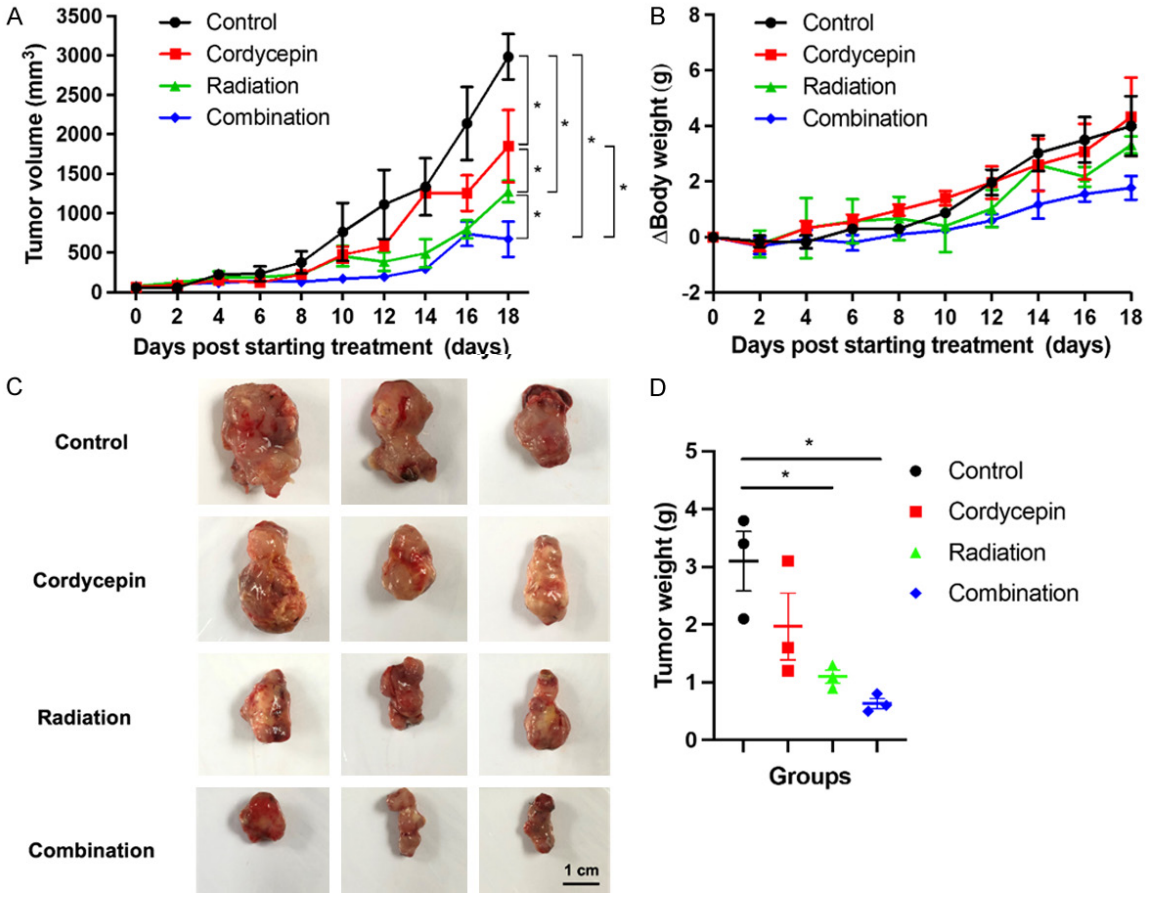

Figure 5. Combination treatment of cordycepin and radiation inhibit MA-10 tumor growth in mice. C57BL/6 mice (N = 3) were subcutaneously inoculated with 1 × 10^6 MA-10 cells in the back region and then treated with radiation alone (4 Gy, single dose) or cordycepin alone (20 mg/kg, intraperitoneally, every other day) or in combination. The tumor volume (A) and body weight (B) were measured every other day. Mice were sacrificed on the 18th day post treatment. The size (C) and weight (D) of transplanted tumors among different groups were examined. $^*P$<0.05 represents significantly statistical difference between different groups.

(**Figure 5B**). Furthermore, similar patterns as tumor volume data can be observed in the size and weight of tumors removed after mice sacrifice. As expected, combination treatment displayed the most pronounced suppression of MA-10 tumor size and weight (P<0.05) (**Figure 5C** and **5D**). These results illustrate that the combination treatment with cordycepin and radiation can effectively inhibit the mouse MA-10 tumor growth and its curative effect is better than that of cordycepin or radiation alone.

Combination treatment reduced γ-H2AX expression in mouse transplanted MA-10 tumor

To further clarify that the inhibition of tumor growth in mice is mediated via the DNA damage caused by the combination treatment, immunohistochemistry (IHC) examination of γ-H2AX was carried out. The results demonstrated that the expression levels of γ-H2AX were significantly increased in the tumor tissues obtained from cordycepin alone-, radiation alone-, and the combination treatment-treated mice showing by the dark brown color area and the deeply stained cell numbers increased, when compared to the levels in the tumor tissues obtained from control mice (**Figure 6A**). The image quantitative analysis also showed that the positive area percentages of the cordycepin alone (17%), radiation alone (19%), and combination (30%) treatment groups were significantly increased compared to the control (3%) group (P<0.05) (**Figure 6B**). Furthermore, the expressive strength of γ-H2AX (**Figure 6A**), and the positive area percentage (P<0.05) (**Figure 6B**) of the tumor sections iso-

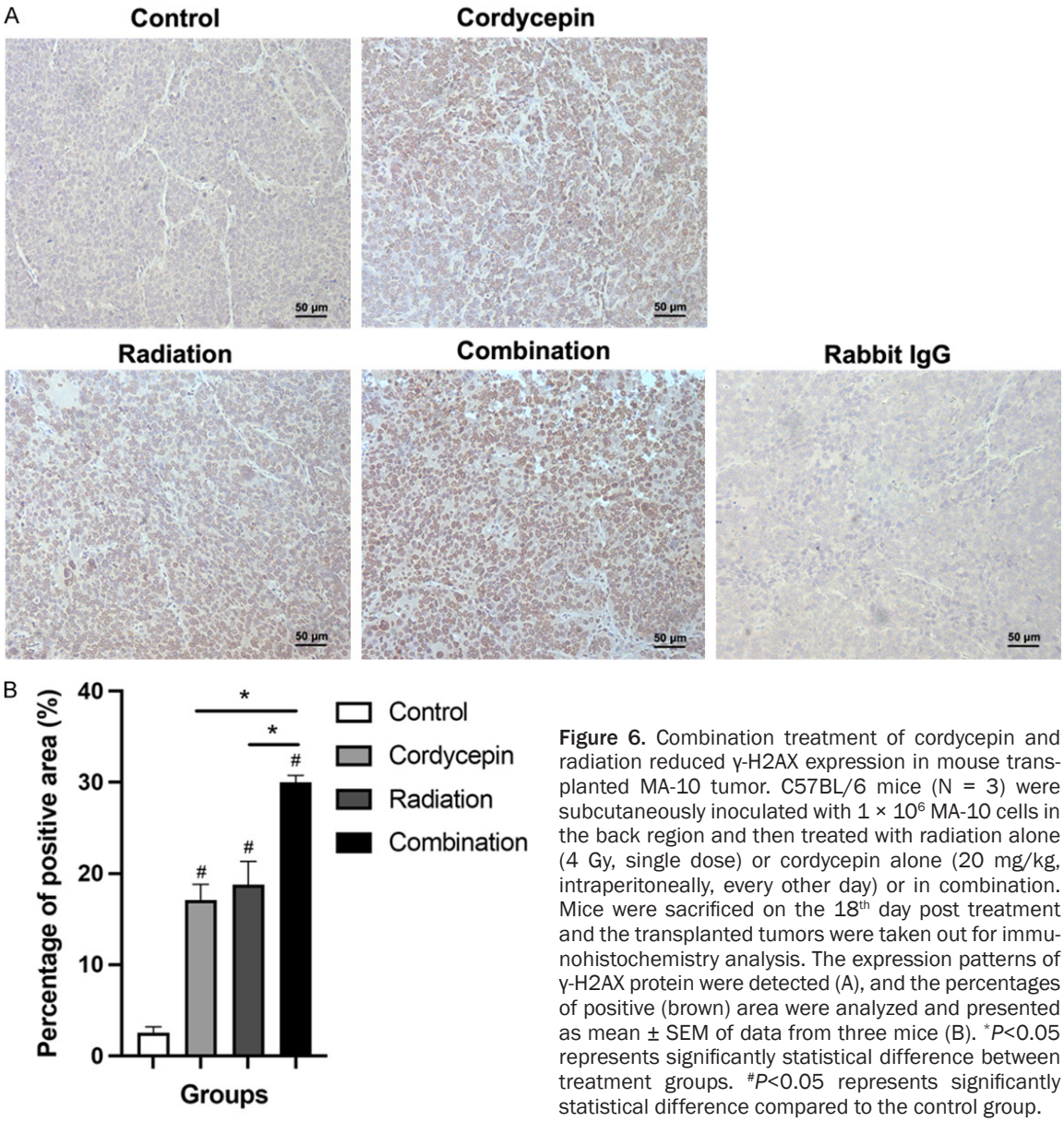

Figure 6. Combination treatment of cordycepin and radiation reduced γ-H2AX expression in mouse transplanted MA-10 tumor. C57BL/6 mice (N = 3) were subcutaneously inoculated with 1×10^6 MA-10 cells in the back region and then treated with radiation alone (4 Gy, single dose) or cordycepin alone (20 mg/kg, intraperitoneally, every other day) or in combination. Mice were sacrificed on the 18th day post treatment and the transplanted tumors were taken out for immunohistochemistry analysis. The expression patterns of γ-H2AX protein were detected (A), and the percentages of positive (brown) area were analyzed and presented as mean ± SEM of data from three mice (B). $^*P<0.05$ represents significantly statistical difference between treatment groups. $^\#P<0.05$ represents significantly statistical difference compared to the control group.

lated from the combination treatment-treated mice were significantly enlarged when compared to other treatment alone groups. These results support that the inhibition of tumor growth by the combination treatment with cordycepin and radiation in mice is highly correlated with the induction of DNA damage in MA-10 tumor cells.

Discussion

In this study, our data demonstrate that both cordycepin and radiation treatment alone can increase intracellular ROS production and cause DNA damage in MA-10 mouse Leydig tumor cells. Combining cordycepin with radiation treatment produces a synergistic effect, as it enhances the accumulation of intracellular ROS and the extent of DNA damage as well as the inhibition of tumor growth *in vivo*, potentially resulting in more tumor destruction. These results echoed our previous study [27] and agreed with those showing that the combination treatment is more effective in anti-tumor effect than cordycepin or radiation treatment alone.

It was recently established that cordycepin could enhance radiosensitivity in cervical cancer cells, breast cancer cells, and oral cancer

cells by prolonging radiation-induced cell cycle G2/M arrest, elevating ROS production via Nrf2 modulation, and inducing autophagy and apoptosis through cell cycle arrest and modulating DNA damage repair, respectively [15, 30-32]. Based on these reports, the synergistic effect of cordycepin and radiation might be a common phenomenon in different tumors. Nonetheless, the finding of present study is highly valuable because of following innovation points. First, a common treatment for testicular tumors including Leydig cell tumors is orchiectomy, which profoundly affects reproductive function. An alternative approach, using cordycepin to enhance Leydig cell radiosensitivity thereby reducing the radiation dose of treatment protocol to kill Leydig tumor cells, but preserving the testis for fully functional reproduction, would be highly appreciated. Second, recent findings by our group showed that cordycepin is able to stimulate normal Leydig cell steroidogenesis [33, 34]. Combine cordycepin into the radiotherapy of testicular cancer not only enhances the radiosensitivity of Leydig tumor cells, but also stimulates normal Leydig cells to increase the synthesis of sex hormones, thereby maintaining normal reproductive function. Therefore, study on antitumor effect and mechanism of the combination treatment of cordycepin and radiation in Leydig tumor have more clinical application value than other cancers. Third, Leydig cell tumor is rare. Thus, there are few related research literatures can be found in this field. The finding of this study may fulfill more valuable information.

Many studies have pointed out that ROS can mediate the response to chemotherapy or radiotherapy by affecting the signaling pathways of survival or death in cells [35-37]. A recent report by our team revealed that cordycepin can enhance the radiosensitivity to induce apoptosis via modulating the cell cycle-, caspase- and ER stress-related signaling pathways in MA-10 cells [27]. Here, we show that the combination treatment can also mediate MA-10 cell death by causing excessive ROS accumulation and DNA damage. These two articles illustrate the detailed molecular mechanism of how combination treatment of cordycepin and radiation kills MA-10 cells. In fact, both cordycepin and radiation can increase intracellular ROS production, and combination treatment will make the accumulation of ROS in MA-10 cells more serious. This may be the reason of why the combination treatment caused more severe DNA damage than that cordycepin alone or radiation alone treatment. In the present study, we analyzed the expression of a ROS regulatory molecule, HO-1, in MA-10 cells after receiving different treatments. In the combination treatment group, the expression of HO-1 was significantly inhibited at 24 hr, which was negative correlated with the production of intracellular ROS (**Figure 1**). However, such association was not clear in the single treatment groups. The inhibition of HO-1 protein in the group that treated with cordycepin alone was more obvious than that treated with radiation alone, but the generation of ROS was opposite (**Figure 1**). This phenomenon suggests that there may be some other regulatory mechanisms that also play a role in the regulation of ROS. Most of cells have their own complex ROS scavenging system including superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase, peroxiredoxin, thioredoxin and catalase, which can convert superoxide anions to water and recover the antioxidants in the reduced state [38]. Whether these ROS regulatory systems are also involved in the ROS induction of MA-10 by the combination treatment of cordycepin and radiation needs to be further clarified.

The accumulation of ROS can further trigger DNA damage and initiate DNA damage-related signal transduction pathways, eventually leading to cell apoptosis [28]. DNA fragmentation is a biochemical hallmark of apoptosis [39]. In the quantitative analysis results of comet assay, the percentages of tail DNA content in both single and combination treatments were much higher than that in the control group, indicating that all treatments could cause DNA damage in MA-10 cells (**Figure 2B**). Interestingly, the level of tail DNA moment (tail length × % of DNA in the tail) in combination treatment group was much higher than those in the single treatment groups and the control group, indicating that the comet events in the combination treatment group have longer tail length than other groups (**Figure 2B**). This phenomenon reflects that the combination treatment triggers an effective cleavage mechanism to cut chromosomal DNA into oligonucleosomal size fragments resulting in DNA fragmentation showing in the comet tails. Furthermore, the strategy of adding cordy-

cepin to radiation treatment accelerated the phosphorylation of H2AX, allowing the expression of γ-H2AX in advance (**Figure 2C** and **2D**). γ-H2AX is an adapter molecule in the ATM/Chk2 signaling axis as well, which can lead to the recruitment and accumulation of specific DNA damage response proteins at DNA ends [40]. Thus, phosphorylated Chk2 protein expression was significantly enhanced in MA-10 cells treated with the combination of cordycepin plus radiation at 24 hr, and this superior effect lasted up to 48 hr (**Figure 3C** and **3D**). This outcome may lead to earlier induction of apoptosis in MA-10 cells. Moreover, activation of ATM/Chk2 or ATR/Chk1 signaling axis has been identified that can lead to cell cycle arrest in G2/M phase [41]. Therefore, except the activation of p53-dependent downstream pathway, the activation of ATM/Chk2 or ATR/Chk1 signaling axis can also induce cell death by cell cycle arrest. It has been shown that ROS generation is associated with ER stress activation [16]. Enhanced ROS generation or oxidative stress as we demonstrated in this study might be linked to subsequent ER stress and ER stress-dependent cancer cell apoptosis.

In **Figure 4**, cell viability of MA-10 cells treated with the combination treatment was only partially restored in the presence of NAC, suggesting that MA-10 cell death has other causes besides ROS. In addition to DNA damage caused by ROS, radiation can also directly cause single or double stranded DNA breaks [42]. If it is not repaired in time, the cell will die. Although the exact mechanism by which cordycepin kills cancer cells is still unclear, the possible reasons why it helps radiation therapy achieve better anti-cancer effects can be gleaned from past literature. The major anticancer activity of cordycepin is likely to be mediated by cell surface receptors such as adenosine receptors (ADORAs), death receptors (DRs), and the epidermal growth factor receptor (EGFR). After engaging with the receptors, it in turn to affect various types of cells, tissues, and organ systems, via both intracellular and extracellular signaling pathways such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK), caspases and glycogen synthase kinase-3 beta (GSK-3β) signaling pathways, ultimately causes cell cycle arrest and ER stress to induce apoptosis [43, 44]. Cordycepin can also enhance the effect of radiation-induced cell cycle arrest by blocking it in G2/M phase and down-regulate the anti-apoptotic Bcl-2 protein expression to promote apoptosis [27]. Furthermore, cordycepin might reduce poly(A) tails as a polyadenylation inhibitor of some apoptosis regulatory molecules, finally magnify the radiation-induced apoptosis [45].

The mechanism of inhibition of tumor growth by combination treatment *in vivo* may be more complex. Except directly causing DNA damage to induce Leydig tumor cell death through the overproduction of ROS, angiogenesis might also be involved in the synergistic effect of the combination treatment of cordycepin and radiation. Our previous report indicated that an angiogenic marker, CD31, were significantly decreased in tumor tissues obtained from the cordycepin plus radiation-treated mice [27]. In fact, the ability of cordycepin to inhibit angiogenesis has been explored in past studies. It has been reported that cordycepin is able to suppress angiogenetic capabilities via down-regulation of focal adhesion kinase and induction of p53 and p21 in endothelial cells [46]. In addition, cordycepin also has potential anti-angiogenetic effect on hepatocellular carcinoma and cholangiocarcinoma both *in vivo* and *in vitro* [47, 48]. The causal relationship and related mechanisms of combination treatment of cordycepin and radiation on angiogenesis in the tumor microenvironment still need to be further explored.

Cordycepin has been under some studies to evaluate the potential benefits in cancer therapies as a part of combinational treatments. Apart from the combination with radiotherapy, cordycepin could enhance the chemosensitivity of esophageal cancer cells, head and neck tumor cells, oral cancer cells, osteosarcoma, as well as MA-10 mouse Leydig tumor cells to cisplatin by inducing the activation of AMP activated protein kinase (AMPK) and suppressing the protein kinase B (PKB or AKT) signaling pathway or promoting apoptotic effect through caspase/MAPK pathways [49-53]. Cordycepin could also reverse cisplatin resistance in human bladder cancer cells and non-small cell lung cancer by inactivating ETS proto-oncogene 1 (Ets-1) dependent multidrug resistance 1 gene (MDR1) transcription and activating

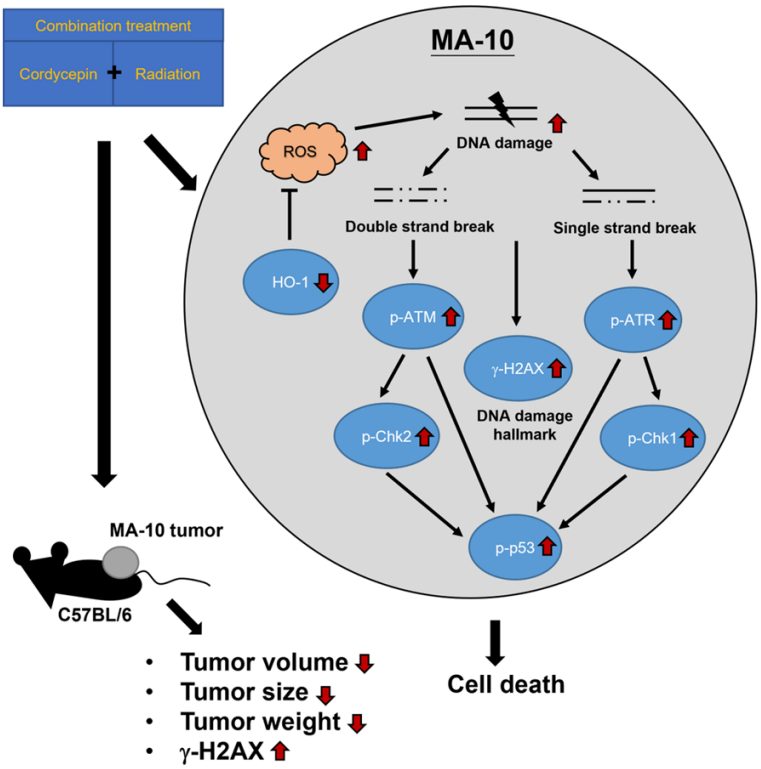

Figure 7. Schematic representation of the combination treatment of cordycepin and radiation inducing MA-10 cell death through ROS production and DNA damage. Combination treatment of cordycepin and radiation leads to ROS accumulation by suppressing the expression of HO-1 protein in MA-10 mouse Leydig tumor cells. It may act directly or indirectly through intracellular ROS to cause DNA damage in MA-10 cells, resulting in an increase in the expression of a DNA damage hallmark, γ-H2AX. DNA double stranded break, or single stranded break will further induce phosphorylation of ATM and Chk2 proteins, or ATR and Chk1 proteins, respectively. The activation of ATM, ATR, Chk1, and Chk2 can all initiate p53-dependent signal transduction pathways and eventually induce cell death. In C57BL/6 mouse MA-10 tumor model, the combination treatment of cordycepin and radiation effectively inhibits tumor growth as reflected in the reduction of tumor volume, size and weight, and high expression level of γ-H2AX in tumor tissue, suggesting that the combination therapy inhibits tumor growth by causing DNA damage in MA-10 Leydig tumor cells.

AMPK/inhibiting AKT signaling pathway, respectively [54, 55]. Moreover, the combination of cordycepin with 5-fluorouracil sensitized chemo-resistant K562 cells to apoptosis through polyadenylation inhibition and thus polyadenylation could prove to be a fine target for overcoming drug resistance [56]. Therefore, it is very potential to use cordycepin as an enhancer in combination with the current cancer therapy protocols in the future.

In summary, the combination treatment of 25 μM cordycepin and 4 Gy radiation effectively causes the intracellular ROS overproduction in MA-10 mouse Leydig tumor cells through the downregulation of HO-1 protein expression. The accumulation of ROS further triggers DNA damage and activates DNA damage-related signal transduction pathways, ATM/Chk2 and ATR/Chk1, as well as increases the expression of DNA damage hallmark, γ-H2AX. The p53-dependent signaling pathway is activated eventually and leads to MA-10 cell death. In mouse model, the combination treatment is also able to effectively inhibit the growth of MA-10 tumors by inducing DNA damage in MA-10 cells (**Figure 7**). Our findings revealed the feasibility and molecular mechanism behind the combined of cordycepin into radiotherapy in the treatment of Leydig cell tumor. This breakthrough is very valuable for improving the clinical treatment protocol of testicular cancer in the future.

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Disclosure of conflict of interest

None.

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References

[1] Young RH. Testicular tumors--some new and a few perennial problems. Arch Pathol Lab Med 2008; 132: 548-564.

[2] Al-Agha OM and Axiotis CA. An in-depth look at Leydig cell tumor of the testis. Arch Pathol Lab Med 2007; 131: 311-317.

[3] Kong Q, Yu Y, Tian T, Zhang H, Sun M, Liu R and Liu Y. Leydig cell tumor of a testis with azoospermia: a case report and literature review. Medicine (Baltimore) 2020; 99: e22085.

[4] Kliesch S. Diagnosis and treatment of Leydig cell tumors. Urologe A 2021; 60: 880-885.

[5] Baskar R, Lee KA, Yeo R and Yeoh KW. Cancer and radiation therapy: current advances and future directions. Int J Med Sci 2012; 9: 193-199.

[6] Azzam EI, Jay-Gerin JP and Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett 2012; 327: 48-60.

[7] Jia S, Ge S, Fan X, Leong KW and Ruan J. Promoting reactive oxygen species generation: a key strategy in nanosensitizer-mediated radiotherapy. Nanomedicine (Lond) 2021; 16: 759-778.

[8] Rowley MJ, Leach DR, Warner GA and Heller CG. Effect of graded doses of ionizing radiation on the human testis. Radiat Res 1974; 59: 665-678.

[9] Izard MA. Leydig cell function and radiation: a review of the literature. Radiother Oncol 1995; 34: 1-8.

[10] Shashidhar MG, Giridhar P, Udaya Sankar K and Manohar B. Bioactive principles from cordyceps sinensis: a potent food supplement - a review. J Funct Foods 2013; 5: 1013-1030.

[11] Tan L, Song X, Ren Y, Wang M, Guo C, Guo D, Gu Y, Li Y, Cao Z and Deng Y. Anti-inflammatory effects of cordycepin: a review. Phytother Res 2020; 35: 1284-1297.

[12] Yang LY, Chen A, Kuo YC and Lin CY. Efficacy of a pure compound H1-A extracted from Cordyceps sinensis on autoimmune disease of MRL lpr/lpr mice. J Lab Clin Med 1999; 134: 492-500.

[13] Zheng Q, Sun J, Li W, Li S and Zhang K. Cordycepin induces apoptosis in human tongue cancer cells in vitro and has antitumor effects in vivo. Arch Oral Biol 2020; 118: 104846.

[14] Pan BS, Wang YK, Lai MS, Mu YF and Huang BM. Cordycepin induced MA-10 mouse Leydig tumor cell apoptosis by regulating p38 MAPKs and PI3K/AKT signaling pathways. Sci Rep 2015; 5: 13372.

[15] Dong J, Li Y, Xiao H, Luo D, Zhang S, Zhu C, Jiang M, Cui M, Lu L and Fan S. Cordycepin sensitizes breast cancer cells toward irradiation through elevating ROS production involving Nrf2. Toxicol Appl Pharmacol 2019; 364: 12-21.

[16] Redza-Dutordoir M and Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta 2016; 1863: 2977-2992.

[17] Loboda A, Damulewicz M, Pyza E, Jozkowicz A and Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell Mol Life Sci 2016; 73: 3221-3247.

[18] Chiang SK, Chen SE and Chang LC. The role of HO-1 and its crosstalk with oxidative stress in cancer cell survival. Cells 2021; 10: 2401.

[19] Maynard S, Schurman SH, Harboe C, de Souza-Pinto NC and Bohr VA. Base excision repair of oxidative DNA damage and association with cancer and aging. Carcinogenesis 2009; 30: 2-10.

[20] Chatterjee N and Walker GC. Mechanisms of DNA damage, repair, and mutagenesis. Environ Mol Mutagen 2017; 58: 235-263.

[21] Yang J, Yu Y, Hamrick HE and Duerksen-Hughes PJ. ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. Carcinogenesis 2003; 24: 1571-1580.

[22] Mladenov E, Fan X, Dueva R, Soni A and Iliakis G. Radiation-dose-dependent functional synergisms between ATM, ATR and DNA-PKcs in checkpoint control and resection in G(2)-phase. Sci Rep 2019; 9: 8255.

[23] Heinrichs S and Deppert W. Apoptosis or growth arrest: modulation of the cellular response to p53 by proliferative signals. Oncogene 2003; 22: 555-571.

[24] Kruse JP and Gu W. Modes of p53 regulation. Cell 2009; 137: 609-622.

[25] Smith HL, Southgate H, Tweddle DA and Curtin NJ. DNA damage checkpoint kinases in cancer. Expert Rev Mol Med 2020; 22: e2.

[26] Bieging KT, Mello SS and Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer 2014; 14: 359-370.

[27] Lee YP, Huang WR, Wu WS, Wu YH, Ho SY, Wang YJ and Huang BM. Cordycepin enhances radiosensitivity to induce apoptosis through cell cycle arrest, caspase pathway and ER stress in MA-10 mouse Leydig tumor cells. Am J Cancer Res 2022; 12: 3601-3624.

[28] Srinivas US, Tan BWQ, Vellayappan BA and Jeyasekharan AD. ROS and the DNA damage response in cancer. Redox Biol 2019; 25: 101084.

[29] Kinner A, Wu W, Staudt C and Iliakis G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res 2008; 36: 5678-5694.

[30] Seong da B, Hong S, Muthusami S, Kim WD, Yu JR and Park WY. Cordycepin increases radiosensitivity in cervical cancer cells by overriding or prolonging radiation-induced G2/M arrest. Eur J Pharmacol 2016; 771: 77-83.
[31] Ho SY, Wu WS, Lin LC, Wu YH, Chiu HW, Yeh YL, Huang BM and Wang YJ. Cordycepin enhances radiosensitivity in oral squamous carcinoma cells by inducing autophagy and apoptosis through cell cycle arrest. Int J Mol Sci 2019; 20: 5366.
[32] Su NW, Wu SH, Chi CW, Tsai TH and Chen YJ. Cordycepin, isolated from medicinal fungus cordyceps sinensis, enhances radiosensitivity of oral cancer associated with modulation of DNA damage repair. Food Chem Toxicol 2019; 124: 400-410.
[33] Leu SF, Poon SL, Pao HY and Huang BM. The in vivo and in vitro stimulatory effects of cordycepin on mouse Leydig cell steroidogenesis. Biosci Biotechnol Biochem 2011; 75: 723-731.
[34] Pao HY, Pan BS, Leu SF and Huang BM. Cordycepin stimulated steroidogenesis in MA-10 mouse Leydig tumor cells through the protein kinase C pathway. J Agric Food Chem 2012; 60: 4905-4913.
[35] Fan PC, Zhang Y, Wang Y, Wei W, Zhou YX, Xie Y, Wang X, Qi YZ, Chang L, Jia ZP, Zhou Z, Guan H, Zhang H, Xu P and Zhou PK. Quantitative proteomics reveals mitochondrial respiratory chain as a dominant target for carbon ion radiation: delayed reactive oxygen species generation caused DNA damage. Free Radic Biol Med 2019; 130: 436-445.
[36] Zulato E, Ciccarese F, Agnusdei V, Pinazza M, Nardo G, Iorio E, Curtarello M, Silic-Benussi M, Rossi E, Venturoli C, Panieri E, Santoro MM, Di Paolo V, Quintieri L, Ciminale V and Indraccolo S. LKB1 loss is associated with glutathione deficiency under oxidative stress and sensitivity of cancer cells to cytotoxic drugs and γ-irradiation. Biochem Pharmacol 2018; 156: 479-490.
[37] Ma Y, Chapman J, Levine M, Polireddy K, Drisko J and Chen Q. High-dose parenteral ascorbate enhanced chemosensitivity of ovarian cancer and reduced toxicity of chemotherapy. Sci Transl Med 2014; 6: 222ra218.
[38] Perillo B, Di Donato M, Pezone A, Di Zazzo E, Giovannelli P, Galasso G, Castoria G and Migliaccio A. ROS in cancer therapy: the bright side of the moon. Exp Mol Med 2020; 52: 192-203.
[39] Zhang JH and Xu M. DNA fragmentation in apoptosis. Cell Res 2000; 10: 205-211.
[40] Mah LJ, El-Osta A and Karagiannis TC. gammaH2AX: a sensitive molecular marker of DNA damage and repair. Leukemia 2010; 24: 679-686.
[41] Hung SY, Lin SC, Wang S, Chang TJ, Tung YT, Lin CC, Ho CT and Li S. Bavachinin induces G2/M cell cycle arrest and apoptosis via the ATM/ATR signaling pathway in human small cell lung cancer and shows an antitumor effect in the xenograft model. J Agric Food Chem 2021; 69: 6260-6270.
[42] Wang JS, Wang HJ and Qian HL. Biological effects of radiation on cancer cells. Mil Med Res 2018; 5: 20.
[43] Yoon SY, Park SJ and Park YJ. The anticancer properties of cordycepin and their underlying mechanisms. Int J Mol Sci 2018; 19: 3027.
[44] Radhi M, Ashraf S, Lawrence S, Tranholm AA, Wellham PAD, Hafeez A, Khamis AS, Thomas R, McWilliams D and de Moor CH. A systematic review of the biological effects of cordycepin. Molecules 2021; 26: 5886.
[45] Thomadaki H, Scorilas A, Tsiapalis CM and Havredaki M. The role of cordycepin in cancer treatment via induction or inhibition of apoptosis: implication of polyadenylation in a cell type specific manner. Cancer Chemother Pharmacol 2008; 61: 251-265.
[46] Lin YT, Liang SM, Wu YJ, Wu YJ, Lu YJ, Jan YJ, Ko BS, Chuang YJ, Shyue SK, Kuo CC and Liou JY. Cordycepin suppresses endothelial cell proliferation, migration, angiogenesis, and tumor growth by regulating focal adhesion kinase and p53. Cancers (Basel) 2019; 11: 168.
[47] Lu H, Li X, Zhang J, Shi H, Zhu X and He X. Effects of cordycepin on HepG2 and EA.hy926 cells: potential antiproliferative, antimetastatic and anti-angiogenic effects on hepatocellular carcinoma. Oncol Lett 2014; 7: 1556-1562.
[48] Liu T, Zhu G, Yan W, Lv Y, Wang X, Jin G, Cui M, Lin Z and Ren X. Cordycepin inhibits cancer cell proliferation and angiogenesis through a DEK interaction via ERK signaling in cholangiocarcinoma. J Pharmacol Exp Ther 2020; 373: 279-289.
[49] Gao Y, Chen DL, Zhou M, Zheng ZS, He MF, Huang S, Liao XZ and Zhang JX. Cordycepin enhances the chemosensitivity of esophageal cancer cells to cisplatin by inducing the activation of AMPK and suppressing the AKT signaling pathway. Cell Death Dis 2020; 11: 866.
[50] Chen YH, Wang JY, Pan BS, Mu YF, Lai MS, So EC, Wong TS and Huang BM. Cordycepin enhances cisplatin apoptotic effect through caspase/MAPK pathways in human head and neck tumor cells. Onco Targets Ther 2013; 6: 983-998.
[51] Chen YH, Hao LJ, Hung CP, Chen JW, Leu SF and Huang BM. Apoptotic effect of cisplatin and cordycepin on OC3 human oral cancer cells. Chin J Integr Med 2014; 20: 624-632.
[52] Li HB, Chen JK, Su ZX, Jin QL, Deng LW, Huang G and Shen JN. Cordycepin augments the che-

mosensitivity of osteosarcoma to cisplatin by activating AMPK and suppressing the AKT signaling pathway. Cancer Cell Int 2021; 21: 706.
[53] Kang FC, Chen PJ, Pan BS, Lai MS, Chen YC and Huang BM. Apoptotic effect of cordycepin combined with cisplatin and/or paclitaxel on MA-10 mouse Leydig tumor cells. Onco Targets Ther 2015; 8: 2345-2360.
[54] Oh SS, Lee KW, Madhi H, Jeong JW, Park S, Kim M, Lee Y, Han HT, Hwangbo C, Yoo J and Kim KD. Cordycepin resensitizes T24R2 cisplatin-resistant human bladder cancer cells to cisplatin by inactivating Ets-1 dependent MDR1 transcription. Int J Mol Sci 2020; 21: 1710.
[55] Liao XZ, Gao Y, Zhao HW, Zhou M, Chen DL, Tao LT, Guo W, Sun LL, Gu CY, Chen HR, Xiao ZW, Zhang JX, He MF and Lin LZ. Cordycepin reverses cisplatin resistance in non-small cell lung cancer by activating AMPK and inhibiting AKT signaling pathway. Front Cell Dev Biol 2020; 8: 609285.
[56] Lallas GC, Courtis N and Havredaki M. K562 cell sensitization to 5-fluorouracil- or interferon-alpha-induced apoptosis via cordycepin (3'-deoxyadenosine): fine control of cell apoptosis via poly(A) polymerase upregulation. Int J Biol Markers 2004; 19: 58-66.