Carbon Ion Induces Cell Death and G2/M Arrest Through pRb/E2F1Chk2/Cdc2 Signaling Pathway in X-ray Resistant B16F10 Melanoma Cells

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Abstract
To explore the effect of high-LET carbon ion (C-ion) radiation on malignant melanoma, we systematically compared the radiobiological effects of C-ion with that of X-rays in B16F10 melanoma cells. Results showed that C-ion radiation statistically inhibited clonogenic survival capacity of B16F10 melanoma cells. The RBE was 3.7 at D₁₀ levels, meaning 1.0 Gy C-ion should cause the same biological effect as ≥ 3.0 Gy X-rays. In addition, we also observed a stronger proliferation-inhibiting and higher ratio of cell apoptosis and necrosis in B16F10 cells treated with C-ion than X-rays. Moreover, C-ion radiation exhibited stronger and long-lasting G2/M arrest than X-rays. As an underlying mechanism, we speculated that C-ion radiation-induced G2/M block through activating pRb/E2F1/Chk2 pathway. With these results, we highlighted the potential of C-ion in treatment of cutaneous melanoma. Further, in vitro experiments as well as clinical trials are needed to further evaluate the effect of carbon ion radiotherapy in melanoma.

Keywords
melanoma, carbon ion, cell death, G2/M arrest, pRb/E2F1/Chk2

Introduction
Melanoma, also known as malignant melanoma, arises through malignant transformation of melanocytes. The vast majority of melanoma occurs in the skin, namely cutaneous melanoma. Cutaneous melanoma is the most common type of melanoma, with accounting for 85% of diagnosed cases. Worldwide, approximately 324,000 (1.7%) of all newly diagnosed primary malignancies were cutaneous melanomas.¹ It is the most aggressive and lethal form of all skin cancers. Although it represents approximately 5% of all cutaneous malignancies, it is responsible for the vast skin cancer-related deaths.²,³ Melanoma with early stages can be cured with surgical excision, but a proportion of patients still has a poor prognosis and high mortality due to its strong invasion and metastasis ability.³,⁴

The role of radiotherapy in malignant melanoma is still in discussion. Photon radiotherapy is commonly used especially for the treatment of bone and brain metastases from melanoma.⁵
Different in vitro studies that were published have shown radiotherapy to be able to suppress cell growth in melanoma. However, the range of results reported is heterogeneous. A clear role of radiation therapy in the treatment of primary melanoma has not been well defined. Melanoma cells were considered as intrinsically radioresistant compared to other cancer cells. The broad shoulder on a melanoma survival curve indicated that a higher radiotherapy dose is needed to produce a response. Radioresistance is the major obstacle in the radiotherapy of the malignant melanoma. Accumulating evidence suggests that high linear energy transfer (LET) carbon ion radiotherapy (CIRT) might overcome the relative radioresistance of melanoma through a higher relative biological effectiveness (RBE). The effect of high-LET CIRT was observed in several relative radioresistant tumors, especially in melanoma. Clinical trial has been shown CIRT is able to produce good local control of melanoma. For CIRT, the unique dose profile with Bragg peaks, very steep dose gradients and less low dose exposure due to fewer beams leads to a lower dose to the healthy surrounding tissue. Thus, CIRT is considered one of the superior noninvasive approaches for the treatment of tumors that are resistant to conventional radiotherapy.

Cell cycle checkpoints are supposed to identify the level and type of DNA damage, block cells at an appropriate phase, and activate various systems to repair their damage. Cell-cycle arrest provides an opportunity for the cells to repair DNA damage before entering the mitotic phase. Hence, cell-cycle checkpoints play a critical role in the damaged response system. Unfortunately, most of the cancer cells have mutations in genes involved in the G1 checkpoint such as p53, Rb, p16, MDM2, and cyclin D. Interestingly, the G2 checkpoint is usually retained in the cancer cells with impaired G1 checkpoint. The fact that the radiation-induced G2-phase arrest is a universal event in tumor cells, which renders the G2/M checkpoint as target for improved efficacy of radiation therapy. Furthermore, experimental studies have indicated that the high-LET of carbon ion (C-ion) induces complex DNA damage patterns with difficult to repair clustered DNA double-strand breaks (DSB). The extent of DNA damage determines whether to extend cell cycle arrest or abrogate the check points, leaving options for cellular machinery to repair and subsist or to give up and culminate in a death. Although, in our previous studies, we have found that C-ion radiation induced B16F10 melanoma cells apoptosis and decreased tumor volume, resulting in the median survival time of tumor-bearing mice was significantly improved. However, the effect of C-ion radiation on cell cycle in B16F10 cell line and its molecular mechanisms are currently unknown. It remains to be elucidated whether the cell cycle and cell death induced by C-ion radiation is different from that by X-rays radiation. Thus, in this present study, we evaluated the effect of C-ion as well as X-rays radiation on B16F10 melanoma cells and investigated the alteration of the cell cycle and its possible molecular mechanisms.

Materials and Methods

Cell Culture

Murine B16F10 cutaneous melanoma cells were purchased from the National Collection of Authenticated Cell Culture (Shanghai, China). The cells were cultured in RPMI-1640 (Gibco Life Technologies, Carsbad, USA) medium supplemented with 10% (v/v) fetal bovine serum (HyClone, GE Healthcare Life Sciences, Logan, USA) and 1% antibiotic-antimycotic liquid (Thermo Fisher Scientific Inc.) at 37°C in a humidified atmosphere with 5% CO₂.

Radiation Treatment

The B16F10 cells were seeded in 25 cm² flasks (Corning, 430168) in a suitable number to provide exponential growth of cells at the time of radiation. The cells were exposed to X-rays or C-ion beams at room temperature. X-rays radiation: A 6 MeV X-rays beams (PXi precision X-RAD 225, 380 kV, 300 mA) was used and the dose rate was 4 Gy/min. C-ion radiation: C-ion radiation was performed at the Heavy Ion Research Facility (HIRF) in Lanzhou, China. C-ion beams was delivered in the middle of the 30 mm-extended Bragg peak with energy of 100 MeV/n (linear energy transfer, LET 35.17 keV/μm) and the dose rate was 2 Gy/min.

Clonogenic Survival Assay

After radiation exposure, B16F10 cells were trypsinized and numbered. Thousand cells were seeded in per 60 mm dish. After 8 days, the colonies were fixed with 4% paraformaldehyde for 30 min and stained with 0.01% crystal violet (Sigma–Aldrich) for 15 min at room temperature. Any colonies that generated more than 50 cells were considered clonogenic survivors and the number of colonies was normalized based on each uniradiated control. Data are presented as the mean ± S.D. of at least three independent experiments in triplicate.

Relative Biological Effectiveness (RBE)

The RBEs for B16F10 cells were calculated as the ratio of the \( D_{10} \) and \( D_{50} \) ’s to that of X rays under equal set-conditions. The \( D_{10} \) and \( D_{50} \) values were recalculated from the \( \alpha \) and \( \beta \) terms of the survival data for B16F10 cells exposed to carbon ion beams. The \( \alpha \) and \( \beta \) parameters were obtained from survival data by curve fitting using \( SF = \exp(-\alpha D-\beta D^2) \), where SF is the survival fraction, and D is the radiation dose. The cell susceptibility of cells was described as the inverse value of survival for a given dose of exposure from \( \alpha \) and \( \beta \) parameters for each survival data set.

Cell Apoptosis and Necrosis Assays

After radiation for 24 and 48 hours, B16F10 cells were harvested and stained with FITC-Annexin V and propidium iodide (PI) to analyze the apoptotic/necrotic cells using...
Annexin V/PI staining kit (BD Biosciences, USA). Cell apoptosis and necrosis were measured by BD FACSVerse and analyzed by BD FlowJo® (BD Biosciences, USA).

**Cell Viability and Proliferation Assays**

The irradiated B16F10 cells (5×10³ cells/well) were seeded in 96-well plate and incubated at 37°C in humidified incubator with 5% CO₂ for 24, 48, and 72 hours. The MTS assay was performed using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI, USA).

**Cell Cycle Assay**

The cell cycle distributions were analyzed using flow cytometry. B16F10 cells were first irradiated with the indicated doses of X-rays and C-ion beams. At 6, 12, 24, 48, and 72 h post-radiation, the cells were dispersed using EDTA-free trypsin, washed with phosphate-buffered saline (PBS), then fixed using prechilled 75% ethanol, and stored at -20°C overnight. Fixed cells were centrifuged, washed, and incubated in RNase and propidium iodide prior to measurement of DNA-content using a BD FACSVerse (BD Biosciences, USA). The minimum of 10⁴ ungated cells were analyzed using BD CellQuest Pro 4.0.2 (BD, Biosciences, U.S.A.) and the cell cycle phases were evaluated using ModFit LT 3.0 (Verity Software House, Inc., U.S.A.).

**Western Blot Analysis**

The irradiated B16F10 cells were lysed using RIPA buffer containing 1% PMSF (Beyotime, Shanghai, China) on ice. Concentration of total protein was determined by bicinchoninic acid protein assay (Thermo, USA). 20 μg total proteins were loaded onto 10% SDS-PAGE and then transferred to PVDF membrane (Millipore, IPVH00010), blocked with 0.05% Tween and 5% BSA (bovine serum albumin, BSA) (BBI life sciences Corporation, Canada) in Tris-buffered saline at room temperature. Followed by incubation with primary antibodies: Rb (ab181616), p-Rb (ab184796), E2F1(ab112580), Chk2 (ab59408), Cdc2 (sc53217), p-Cdc2 (sc136014), CyclinB1 (sc7393), and β-actin (AP0060) overnight at 4°C. The preparative membranes were reacted with appropriate secondary antibodies conjugated to HRP. The immunological complexes were visualized using electrochemiluminescence (Millipore, Darmstadt, Germany). The protein brands were detected using Al680 (Alpha Innotech Corporation) and quantified using Image Quant TL software (version 8.1; GE Healthcare Life Sciences).

**Statistical Analysis**

Results are presented as the mean ± standard deviation (SD). Significant differences in means between two samples were analyzed using two-tailed Student’s t-tests and two-way ANOVA. P < 0.05 was considered statistically significant.

**Results**

**Effect of C-Ion Radiation on the Clonogenic Survival Capacity of B16F10 Cells and RBE**

To confirm the radiation sensitivity of B16F10 cells to C-ion or X-rays, we measured the clonogenic survival capacity. Here, we observed the markedly reduced survival capacity of B16F10 cells at 8 days after C-ion radiation compared to that of X-rays (Figure 1A). The surviving fraction for C-ion radiation showed a decrease in a dose-dependent manner. Although the curve with X rays showed a big shoulder (Figure 1B). The D₁₀ values were 7.5 and 2.1 Gy for X-rays and C-ion, respectively. And the RBE at D₁₀ level was 3.57. The D₅₀ values were 3.1 and 0.6 Gy for X-rays and C-ion, respectively. The RBE at D₅₀ level was 5.16 (Figure 1B). This result suggested that C-ion beams have a higher killing effect on B16F10 cells than X-rays.

**C-Ion Radiation Induced More Cell Apoptosis and Necrosis in B16F10 Melanoma Cells Than X-Rays**

To investigate if this killing effect was reflected by the cell apoptosis and necrosis, we measured the cells death using Flow cytometry. The apoptosis and necrosis of B16F10 melanoma cells were reduced after radiation in dose-depend manner, which was more prominent for C-ion beams than X-rays at all dose points (Figure 2). The apoptosis and necrosis induced by C-ion significantly was heavier than that by X-rays at 24 h (Figure 2A and 2B) and 48 h (Figure 2C and 2D) after radiation. X-rays showed less killing effects and rather decreased the cell apoptosis and necrosis at 0.25 Gy and 0.5 Gy compared to non-irradiated control (Figure 2B and 2D). The cell apoptosis and necrosis were significantly induced by ≥ 2.5 Gy X-rays, as well as ≥ 0.5 Gy C-ion beams (Figure 2B and 2D). The killing effects reflected by the cell apoptosis and necrosis assay suggested that using a radiation dose of 2.5 Gy of C-ions, and the same experiment was conducted using 7.5 Gy of X-rays for comparison (Figure 2B and 2D). The apoptotic and necrotic cells were ~18.9% for 7.5 Gy X-rays and ~19.3% for 2.5 Gy C-ions 24 h after radiation, ~23.6% for 7.5 Gy X-rays, and ~21.7% for 2.5 Gy C-ions 48 h after radiation.

**Carbon Ion Beams Radiation Exhibits Stronger Proliferation-Inhibiting Effect Than X-rays in B16F10 Melanoma Cells**

To further evaluate the effects of C-ion beams and X-rays on cell viability and proliferation, cytotoxicity, and proliferation assay were performed using MTS assay. As Figure 3 showed, the cell viability was inhibited by both types of radiation in
Figure 1. Carbon ions radiation is more effective in suppressing clonogenic survivor than X-rays. (A) Clonogenic survival capacity of B16F10 cells. (B) Curve of survival fraction. Symbols indicate mean and error bars SD values. Lines show fits according to the nonlinear-quadratic model Exp3P2. The relative biological effectiveness (RBE) of carbon ions radiation compared to X-rays is indicated at a survival level of 50% (RBE50) and 10% (RBE10).

Figure 2. Carbon ions radiation induces higher ration of cell apoptosis and necrosis than X-rays in B16F10 melanoma cells. (A) and (C) Representative dot plots showing apoptotic and necrotic cells. Apoptosis and necrosis were investigated by flow cytometry 24 h and 48 h after radiation, respectively. (B) and (D) The percent of apoptotic and necrotic cells 24 h and 48 h after irradiation, respectively. **p ≤ 0.01, ***p ≤ 0.001 vs. Control group; ###p ≤ 0.01, #####p ≤ 0.001 vs. X-ray radiation.
dose-dependent manner, and C-ion beams radiation exhibited a stronger inhibitory effect than that of X-rays (Figure 3A). The 7.5 Gy X-rays radiation showed a comparable proliferation-inhibition to 2.5 Gy C-ions, but B16F10 cells treated with 7.5 Gy X-rays restored growth 48 h after radiation (Figure 3B). The 0.5-7.5 Gy X-rays radiations showed a wide range of proliferation-inhibition, while C-ion beams showed greater inhibitory effects (Figure 3C-D). The B16F10 melanoma cells treated with 7.5 Gy C-ion beams could not return growth even 96 h after radiation (Figure 3D). These results indicated that high dose of C-ion-induced cell viability inhibition lasted for ≥96 h after radiation, while the inhibition of X-rays lasted for only 48 h. C-ion radiation exhibited stronger and longer-lasting inhibitory effect on cell viability and proliferation than that of X-rays.

C-Ion Radiation Increases G2/M Arrest Compared to X-Rays

Figure 4 showed cells distribution in cell cycle at different radiation dose and time points after radiation. The G2/M phase increased in dose-dependent manner at 24 h after exposure to X-rays or C-ions (Figure 4A). The 7.5 Gy X-rays radiation showed a comparable proliferation-inhibition to 2.5 Gy C-ions, but B16F10 cells treated with 7.5 Gy X-rays restored growth 48 h after radiation (Figure 4B). The 0.5-7.5 Gy X-rays radiations showed a wide range of proliferation-inhibition, while C-ion beams showed greater inhibitory effects (Figure 4C-D). The B16F10 melanoma cells treated with 7.5 Gy C-ion beams could not return growth even 96 h after radiation (Figure 4D). These results indicated that high dose of C-ion-induced cell viability inhibition lasted for ≥96 h after radiation, while the inhibition of X-rays lasted for only 48 h. C-ion radiation exhibited stronger and longer-lasting inhibitory effect on cell viability and proliferation than that of X-rays.

G2/M Phase Arrest Is Mediated by pRb/E2F1/Chk2/Cdc2 Signaling Pathway

To evaluate whether radiation-induced G2/M arrest in B16F10 cells correlated with cell cycle regulators, we examined the levels of checkpoint related proteins using western blotting at indicate dose (Figure 5A and B). An accumulation of Chk2, Cdc2, and cyclin B1 were observed at 24 h post-radiation (Figure 5A), the increased levels were in dose-dependent manner.

Figure 3. The proliferation-inhibiting effect of carbon ions radiation compared to X-rays. (A) Cell viability of B116F10 melanoma cells treated with carbon ion beams or X-rays at the indicated dose 48 h after radiation. (B) Growth curve of cells. B16F10 melanoma cells were evaluated at 24 h, 48 h, 72 h, and 96 h post treatment with 2.5 Gy carbon ions or 7.5 Gy X-rays, carbon ion beams radiation exhibits long-lasting growth inhibitory effects. (C-E) Growth curve of cells at same dose of carbon ions or X-rays, indicating stronger growth inhibitory effects for carbon ions radiation. Raw data was determined in three independent experiments. ***p ≤ 0.001 vs. X-ray radiation.
manner (Figure 5A and B). Interestingly, we observed a significant rise in the levels of Rb, p-Rb, and E2F1 at the same time point (Figure 5A), suggesting p-Rb/E2F1/Chk2 pathway possibly regulated G2/M cell cycle arrest after radiation. To find the time point specificity for the activation of these molecules, we also assessed Cdc2, p-Cdc2, and cyclin B1 levels at different time points. As expected, Cdc2, pCdc2, and cyclin B1 levels significantly increased from 12h after radiation in B16F10 cells treated with 7.5 X-rays or 2.5 Gy C-ion, and the accumulation/activation was maximal at 24 h after radiation and declined thereafter (Figure 5C and D). Notably, C-ion radiation-induced strong and long-lasting G2/M cycle arrest was confirmed by these proteins' accumulation and activation (Figure 5D).

Discussion
Melanoma is a major skin cancer that affects more than 324,000 patients and kills about 57,000 patients annually.
worldwide, which represents one of the major health problems. Currently available treatment options for patients with melanoma include radiotherapy (RT), chemotherapy, targeted therapy, and immunotherapy. Adjuvant therapies were recommended as an option for patients who were diagnosed with unresectable or advanced forms of melanoma. However, the role of conventional photons radiotherapy in melanoma is still in discussion due to its relative resistance to photons radiation, such as X- and γ-rays. Although, up to date, intro and vivo data on CIRT in melanoma is limited, accumulating evidence has suggested that CIRT will be a promising therapeutic option for melanoma patients due to its high physical dose conformity and greater RBE than photons. For example, Aninditha KP, et al. indicated that 12C and 16O heavy ions showed a greater inhibitory effect on cell proliferation compared to X-rays and an increased G2/M arrest in two different cell lines of melanoma. And Saito K., et al. also showed C-ion alone had superior cell-killing effects than γ-rays. Matsumoto, Y., et al. found that even low-LET C-ion radiation was more effective than X-rays radiation in suppressing the formation of metastasis in B16/BL6 cells, rather than low-dose X-rays radiation (0.5 Gy) enhanced metastatic abilities of tumor cells. Moreover, in previous study, we found that high-LET C-ion radiation inhibited tumor growth and metastasis of B16F10 cells in mice. These results suggest that carbon ions radiotherapy might overcome the relative radioresistance of melanoma to photons and may a promising modality in treatment of malignant melanoma.

In this present study, we also identified C-ion radiation has superior cell-killing effects than X-rays. The clonogenic survival assay showed that C-ion radiation was statistically more effective than X-rays. The RBE was 3.7 at D_{10} levels, and 5.2 at D_{50} levels (Figure. 1), meaning 1.0 Gy C-ion should cause the same biological effect as ≥ 3.0 Gy X-rays. C-ion radiation induced higher ratio of cell apoptosis and necrosis (Figure. 2). And the superiority of C-ion compared to X-rays in terms of proliferation-inhibiting (Figure. 3). The G2/M cycle arrest increased with higher dose in both types of radiation modalities. But C-ion radiation exhibits stronger and long-lasting G2/M block than X-rays (Figure. 4). Notably, the dose used to achieve the same effect to X-rays radiation is less using heavy ion therapy (2.5 Gy for C-ion compared to the results of 7.5 Gy X-rays), which means that C-ion might be 3 times more effective than X-rays. With these results we were able to show the potential of CIRT in melanoma. Further, in vitro experiments as well as clinical trials are needed to further evaluate effect of CIRT in melanoma.

Checkpoints in the cell cycle regulate the progression or arrest of the cell cycle in response to DNA damage and allow time for DNA repair. These occur either in late G1, which prevents entry to the S phase, or in late G2, which prevents entry to mitosis. If the cell detects successful repair, the cell cycle can continue, and if it detects irreparable errors, it may undergo apoptosis. If apoptosis signaling fails, the DNA repair errors propagate as mutations. The G2/M checkpoint arrest is prominent after exposure to radiation-induced DNA damage. If cells with incompletely replicated DNA break through G2-arrest to enter mitosis, the condensation of partially replicated sister-chromatids will shatter the DNA to cause mitotic catastrophe. Carbon ions induced more severe DSB damage than photons, which was repaired less efficiently in tumor cells. Our cell cycle analyses showed a clear increased G2/M arrest in dose-dependent manner. The G2/M phase increase might be a prolonged G2/M arrest indicating a

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**Figure 5.** Western Blot analysis of cell cycle regulatory proteins. (A) Western blot and (B) grayscale analysis of the expression levels of Rb, p-Rb, E2F1, Chk2, Cdc2, and cyclin B1 in B16F10 melanoma cells treated indicated dose of carbon ion beams or X-rays 48 h after radiation. (C) Western blot and (D) grayscale analysis of Cdc2, p-Cdc2, and cyclin B1 levels in B16F10 melanoma cells treated with 2.5 Gy carbon ions or 7.5 Gy X-rays at different time points after radiation.
high amount of DNA damage, which caused the cells unable to pass the checkpoint to proliferate and therefore stay in the G2/M phase, then undergo death.34 The comparable results of 2.5 Gy C-ion beams to 7.5 Gy X-rays demonstrated the improved effectiveness of carbon ions in term of G2/M arrest (Figure 4C and D), this means a higher amount of DNA damage after C-ion radiation. In fact, ~42.7% cells radiated with 7.5 Gy C-ion were unable to repair the damaged DNA and eventually underwent death (Figure 2C and D).

C-ion radiation mainly contribute to interruption of the G2/M transition. G2/M block is a complex process that involves many regulatory proteins, including cyclin family proteins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs). Although it has been elucidated multiple signaling pathways are involved in G2/M block regulation, the specific molecular mechanism is still unclear. p53, a famous transcription factor, regulates the cell cycle especially by monitoring G1 and G2/M checkpoints. However, some literatures reported that p53 in human melanoma cells could not regulate target genes related to apoptosis and cycle arrest.35 Previous studies have suggested that p53 can participate in G2/M arrest in p53-mutation or deficient cells. Rb is an important substrate of cyclin-dependent kinases, and once Rb is phosphorylated, the E2F1 protein is released to activate downstream gene expression.36 In this present study, we found a significant increase in the levels of Rb, p-Rb, and E2F1, suggesting p-Rb/E2F1 pathway possibly regulated G2/M cell cycle arrest after radiation in B16F10 cells. In addition, Chk2 is required for maintenance, but not initiation, of G2 arrest induced by DNA damage. Chk2 was shown to synergize with other genes or factors that perpetuate DNA damage repair during the G2/M phase rather than inducing G2/M arrest.37 However, the decision of cells to either remain in the G2/M phase or go through G2 into mitosis requires the activation of Cdc2, whose activity is regulated by synthesis of cyclin B1 and subsequent complex formation.38 In our experiment, we found cyclin B1 expression in C-ion radiation-treated B16F10 cells is triggered by ≥4-fold increase. Chk2 and Cdc2 significant accumulation after radiation and reach the maximum at 24h. C-ion radiation treatment seemed to induce long-lasting Chk2 and Cyclin B1 expression along with a simultaneous Cdc2 activation, probably indicating an irreversible G2/M arrest.

Conclusion

In conclusion, we demonstrated that C-ion radiation is more effective than X-rays for killing B16F10 melanoma cells. Compared to X-rays, C-ion radiation showed greater inhibitory effects cell proliferation and survival capacity, and promoted more cell apoptosis/necrosis and increased G2/M arrest. Moreover, C-ion radiation induced long-lasting G2/M arrest, which may be regulated by pRb/E2F1/Chk2 signaling pathway. Our results offer useful information for assessment of the biological effects of C-ion radiation on malignant melanoma. Further in vitro experiments as well as clinical trials are needed to further evaluate effect of carbon ions radiotherapy in melanoma.

Author Contributions

Sha Li and Aihong Mao conceived and designed this study. Hefa Huang, Mengjie Xing, Jin Qin, Liping Zhang, Chao Zhang, Zhongze Tian performed the experiments. Hong Zhang and Yang Liu commented on previous versions of the manuscript. Xingxin Gao and Rui Zhao performed data analysis. Aihong Mao wrote the draft and Sha Li revised it. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

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