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SHORT COMMUNICATION

Dimethyl Sulfoxide Attenuates Ionizing Radiation-induced Centrosome Overduplication and Multipolar Cell Division in Human Induced Pluripotent Stem Cells

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Centrosomes are important organelles for cell division and genome stability. Ionizing radiation exposure efficiently induces centrosome overduplication via the disconnection of the cell and centrosome duplication cycles. Over duplicated centrosomes cause mitotic catastrophe or chromosome aberrations, leading to cell death or tumorigenesis. Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can differentiate into all organs. To maintain pluripotency, PSCs show specific cellular dynamics, such as a short G₁ phase and silenced cell-cycle checkpoints for high cellular proliferation. However, how exogenous DNA damage affects cell cycle-dependent centrosome number regulation in PSCs remains unknown. This study used human iPSCs (hiPSCs) derived from primary skin fibroblasts as a PSC model to address this question. hiPSCs derived from somatic cells could be a useful tool for addressing the radiation response in cell lineage differentiation. After radiation exposure, the hiPSCs showed a higher frequency of centrosome overduplication and multipolar cell division than the differentiated cells. To suppress the indirect effect of radiation exposure, we used the radical scavenger dimethyl sulfoxide (DMSO). Combined treatment with radiation and DMSO efficiently suppressed DNA damage and centrosome overduplication in hiPSCs. Our results will contribute to the understanding of the dynamics of stem cells and the assessment of the risk of genome instability for regenerative medicine. © 2024 by Radiation Research Society

INTRODUCTION

The centrosome, crucial for cell division and genome stability (1), is comprised of two centrioles and pericentriolar

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material (PCM). Pericentrin, a vital coiled protein in PCM and centrioles, is conserved (2–4). A single centrosome exists in the G₁ phase and duplicates in the S phase, and the duplicated centrosomes are distributed to the daughter cells during mitosis (5, 6). DNA-damage-induced cell cycle checkpoints can disrupt this cycle, leading to centrosome overduplication (7). Excessive centrosomes can cause improper cell division, chromosomal aberrations, and tumorigenesis (8, 9), as observed in various cancers (10). Conversely, excess centrosomes can induce mitotic catastrophe via multipolar cell division, resulting in cell death (11–13).

The centrosome duplication cycle is tightly linked to genome maintenance mechanisms, including DNA repair and cell cycle pathways (14–16). For example, cyclin-dependent kinase (CDK) 2, a key regulator of S phase entry, is essential for initiating centrosome duplication (14). Additionally, a DNA repair protein involved in homologous recombination, breast cancer 1 (BRCA1), functions as an E3 ubiquitin ligase, regulating centrosome number by ubiquitinating γ -tubulin (17, 18). BRCA1 activity in this process relies on other proteins involved in the DNA damage response, such as NBS1 and ATR, which are also required for centrosome number regulation (19); defects in these genes lead to centrosome overduplication. Various genotoxic stresses such as ionizing radiation can trigger centrosome overduplication via the activation of DNA-damage induced cell cycle checkpoints in a dose-dependent manner (20, 21). In our previous study, we found that after radiation exposure, cell death in neural progenitor cells during mammalian embryonic development involves both apoptosis and mitotic catastrophe driven by excessive centrosomes (22). Moreover, high-linear energy transfer (LET) radiations, such as carbon and iron ions, are more effective at inducing mitotic catastrophe compared to low-LET radiations (23).

The biological effects of radiation primarily stem from DNA damage within the cell nucleus which can occur through direct and indirect mechanisms. Radical scavengers like dimethyl sulfoxide (DMSO) offer radioprotective

effects by mitigating the indirect action of hydroxyl (OH) radicals. Low-LET radiation, like gamma rays, and high-LET radiation, such as heavy ions, generate OH radicals through the radiolysis of water. Therefore, DMSO provides radioprotection against low- and high-LET radiation (24, 25).

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can differentiate into various organs, making them valuable for regenerative medicine and basic research. Tissue organoids and differentiated cells derived from PSCs are used to evaluate relative biological effectiveness (RBE) (26, 27). PSCs have a shortened G₁ phase and suppressed cell cycle checkpoints crucial for maintaining pluripotency (28, 29). Notably, cellular differentiation can result from the ectopic expression of the CDK2 inhibitor p21 (30). Despite PSCs exhibiting high expression of DNA repair, cell cycle, and centrosome proteins, their molecular dynamics and activity remain largely unknown.

In this study, we used gamma-ray and carbon-ion sources to irradiate human iPSCs (hiPSCs) as a PSC model and measured the centrosome aberrations as a genome maintenance marker to investigate how radiation affects the genome stability of PSCs. Radiation efficiently induced centrosome overduplication and multipolar cell division in hiPSCs. DMSO treatment suppressed DNA damage and centrosome aberrations in the hiPSCs, suggesting that DMSO exhibits a radioprotective effect on PSCs and is a useful tool to assess genome integrity of PSCs.

MATERIALS AND METHODS

Cell Culture

The human osteosarcoma U2OS cell line was obtained from the American Type Culture Collection (ATCC). Human NB1RGB skin fibroblasts and human 201B7 iPSCs were obtained from the RIKEN BioResource Research Center (Tsukuba, Ibaraki 305-0074, Japan). The iPSCs clone 2 (C2) were derived from NB1RGB cells as previously described (34). Briefly, messenger RNA (mRNA) integration-free methods were performed using the Stemgent® StemRNA™ NM Reprogramming Kit for Reprogramming Adult and Neonatal Human Fibroblasts (Stemgent, Cambridge, MA). A non-modified RNA (NM-RNA) reprogramming cocktail comprised of OSKMNL NM-RNA (Oct4, Sox2, Klf4, cMyc, Nanog, and Lin28 reprogramming factors); E3, K3, and B18 immune evasion factors NM-RNA and NMmicroRNAs were transfected into NB1RGB cells using Lipofectamine® RNAiMAX™ transfection reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA). After transfection, the cell colonies were selected. The C2 iPSCs and 201B7 cells were adjusted to the feeder-free culture medium. The iPSCs were maintained with mTeSR plus (Stem Cell Technologies, Vancouver, Canada) supplemented with penicillin/streptomycin. The ROCK inhibitor Y27632 (Chemscene, NJ) and iMatrix 511 silk (Matrixome, Osaka, Japan) were added for every passage. Neural progenitor cells (NPCs) were derived from iPSCs C2 as previously described (34) and maintained with a neural expansion medium (GIBCO, Thermo Fisher Scientific) supplemented with penicillin/streptomycin. U2OS and NB1RGB cells were maintained in Dulbecco-modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum and penicillin/streptomycin. DMSO was purchased from Nacalai Tesque.

Irradiation

Cells were irradiated with gamma rays (0.5 Gy/min dose rate) with a cobalt-60 source in Chiyoda Technol Cobalt Radiation Center at Tokyo Tech. The Heavy Ion Medical Accelerator in Chiba (HIMAC) (Japan) at the National Institutes for Quantum Science and Technology generated a 290 MeV/n monoenergetic carbon ion beam (13 keV/μm).

Immunofluorescence

The cells were seeded onto an 8-well micro slide chamber (ibidi, Gräfelfing, Germany). After radiation exposure, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton X-100/phosphate-buffered saline with Tween 20 (PBS-T) for 5 min at 4°C. The cells were blocked with 1% BSA/PBS-T at 4°C for 1 h and stained with primary antibodies pericentrin rabbit polyclonal antibody (ratio of 1:1,000; Abcam, Cambridge, UK) and alpha-tubulin mouse monoclonal antibody (ratio of 1:1,000; Proteintech, IL) for 4–24 h at 4°C. After washing with PBS-T, the cells were subsequently incubated with the following secondary antibodies: Alexa-488-conjugated anti-rabbit immunoglobulin IgG, 1:1,000 (Molecular Probes, Carlsbad, CA) and Alexa-555-conjugated anti-mouse IgG, 1:1,000 (Molecular Probes). After washing with PBS-T, the cells were mounted using a mounting medium (DAKO, Carpinteria, CA). Fluorescence was visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). At least 100 cells in each sample were examined.

Statistical Analysis

The values represented the mean and standard error of three independent experiments. Statistical analysis was performed using Microsoft Excel and Graph Pad Prism 10. Data obtained from the γH2AX foci were analyzed using a one-way analysis of variance followed by Tukey's post-hoc test (*P < 0.1 and ****P < 0.0001). The frequencies of centrosome overduplication and multipolar cell division were analyzed using a Student's t-test (*P < 0.5, **P < 0.1, ***P < 0.05 and ****P < 0.01).

RESULTS

Centrosome Overduplication and Multipolar Cell Division after Gamma-ray Irradiation in hiPSCs

First, we irradiated the C2 and 201B7 hiPSCs with 2 Gy of cobalt-60 gamma rays to address the effect of radiation on the centrosome number in PSCs (Fig. 1A and B). After radiation exposure, the cells were incubated for 48 h to allow for centrosome duplication. Subsequently, the cells were fixed and stained with a centrosome marker, pericentrin, and microtubule marker, α-tubulin antibodies (Fig. 1A). Human primary skin NB1RGB fibroblasts, human osteosarcoma U2OS cells, and neural progenitor cells (NPCs) derived from C2 iPSCs were used as the differentiated cell control (Fig. 1B). While non-treated cells exhibited 1% or <1% excess centrosomes, both groups of hiPSCs demonstrated a dramatic increase of centrosome overduplication (C2 cells: 22.7%, 201B7 cells: 15.7%) compared with the other differentiated cells (NB1RGB cells: 1.2%, U2OS cells: 4.7%, NPCs: 4.5%) after irradiation. Since excess centrosomes cause mitotic catastrophe through multipolar cell division (11–13, 31), we calculated the frequency of multipolar cell division in the mitotic cells (Fig. 2A and B). As expected, both hiPSCs exhibited a higher frequency of multipolar cell division than the other differentiated cells (C2: 18.5%, 201B7: 13.2%, NB1RGB: 1.0%, U2OS: 3.0%, NPCs: 2.5%), suggesting that hiPSCs, when compared

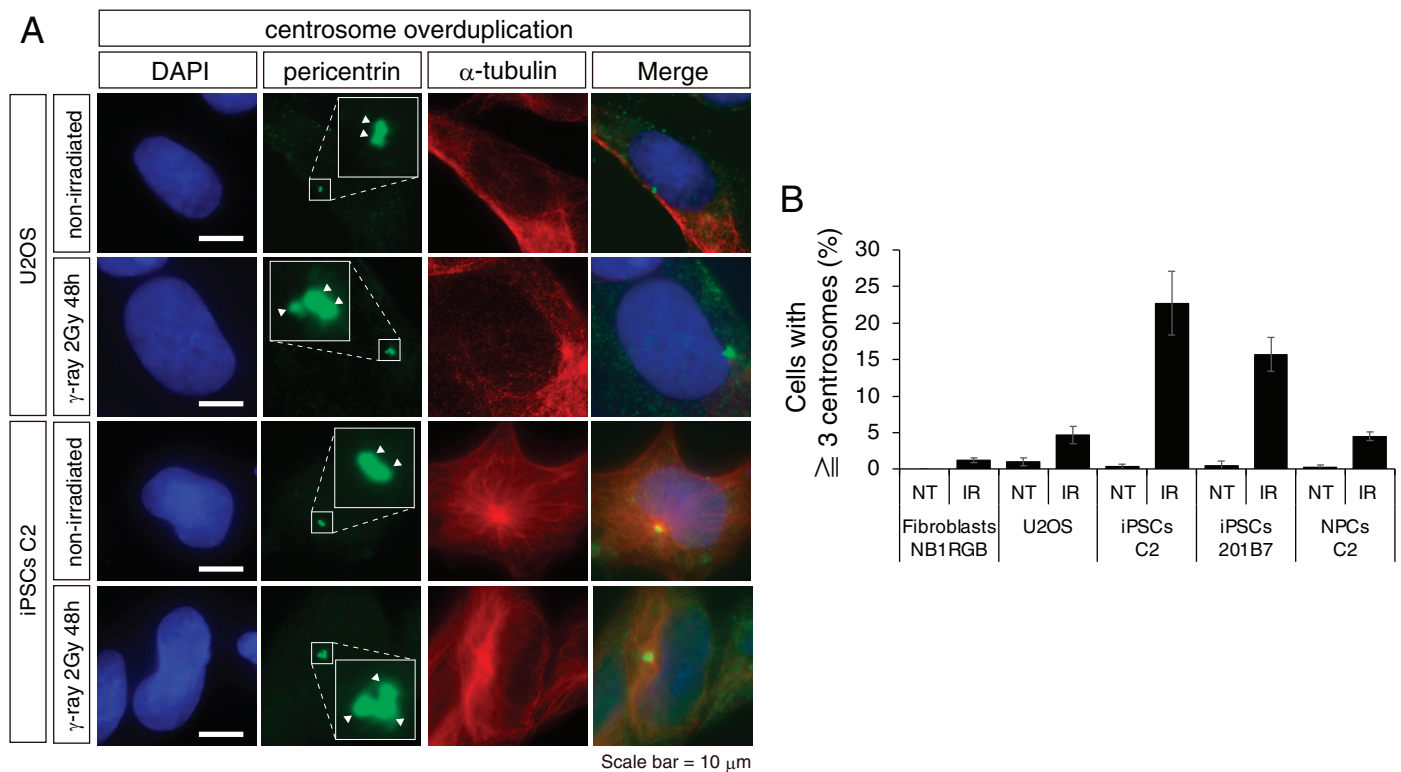


FIG. 1. Centrosome overduplication in hiPSCs and differentiated cells after gamma ray exposure. Panel A: Cells (NB1RGB cells, U2OS cells, C2 iPSCs, 201B7 fibroblasts, and NPCs) were untreated or exposed to 2 Gy of gamma rays and incubated for 48 h. Pericentrin and α -tubulin antibodies were used as centrosome (green) and microtubule markers (red), respectively. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI: blue). Arrowheads indicate centrosomes. Scale bars represent 10 μ m. Panel B: At least 200 cells with overduplicated centrosomes were counted. All experiments were independently performed at least three times. Error bars indicate standard error.

to the other differentiated cells, tend to increase the centrosome number and multipolar cell division after radiation exposure.

Radioprotective Effect of DMSO against DNA Damage in hiPSCs

Low-LET radiation, such as gamma rays, produces free radicals by radiolysis of water. Indirect action by free radicals such as OH radicals is the main source of DNA damage in cells after low-LET irradiation. DMSO is used as a radical scavenger to suppress the indirect effect of low-LET radiation. Subsequently, we analyzed the radioprotective effect of DMSO against DNA damage in PSCs. In pilot experiments, we assessed several DMSO concentrations (0.1 M, 0.2 M, and 0.4 M). High DMSO concentrations exhibited cytotoxicity; cell death occurred in half of the iPSCs after 0.4 M DMSO treatment (data not shown). We used 0.1 M DMSO to prevent unscheduled cytotoxic reactions with DMSO. DMSO (0.1 M) was added to the culture medium 30 min before irradiation, and the cells received 2 Gy gamma-ray irradiation. The cells were fixed and stained with phosphorylated histone H2AX (also known as γ -H2AX) antibody as a marker for DNA double-strand breaks at 30 min incubation postirradiation. The cell nucleus was stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Fig. 3A). We then counted the

number of γ -H2AX foci per nucleus (Fig. 3B). The number of radiation-induced γ -H2AX foci was slightly and markedly attenuated in the PSCs and differentiated cells, respectively (Fig. 4B).

Radioprotective Effect of DMSO against Centrosome Overduplication in hiPSCs

Next, we analyzed the suppressing effect of DMSO against centrosome duplication in hiPSCs. DMSO (0.1 M) was added 30 min prior to irradiation, and the cells were exposed to 2 Gy of gamma rays. After incubation for 48 h, the cells were fixed and stained with pericentrin and α -tubulin antibodies. The frequency of centrosome overduplication in the iPSCs (C2: 22.7% and 201B7: 15.7%) was considerably decreased in the DMSO-containing medium (C2: 11.7%, 201B7: 7.3%) (Fig. 4A), indicating the radioprotective effect of DMSO against centrosome aberrations. The differentiated cells (U2OS cells: 4.7% and NPCs: 4.5%) mildly decreased after DMSO treatment (U2OS cells: 2.7%, NPCs: 3.7%). The frequency of multipolar cell division in the iPSCs (C2: 18.5% and 201B7: 13.2%) also decreased under DMSO treatment (C2: 9.8% and 201B7: 7.7%) (Fig. 4B). In contrast, the differentiated cells (U2OS cells: 3.0%, NPCs: 2.5%) slightly decreased after DMSO treatment (U2OS cells: 2.5%, NPCs: 2.2%). Taken together, DMSO efficiently prevented centrosome overduplication in hiPSCs after radiation exposure.

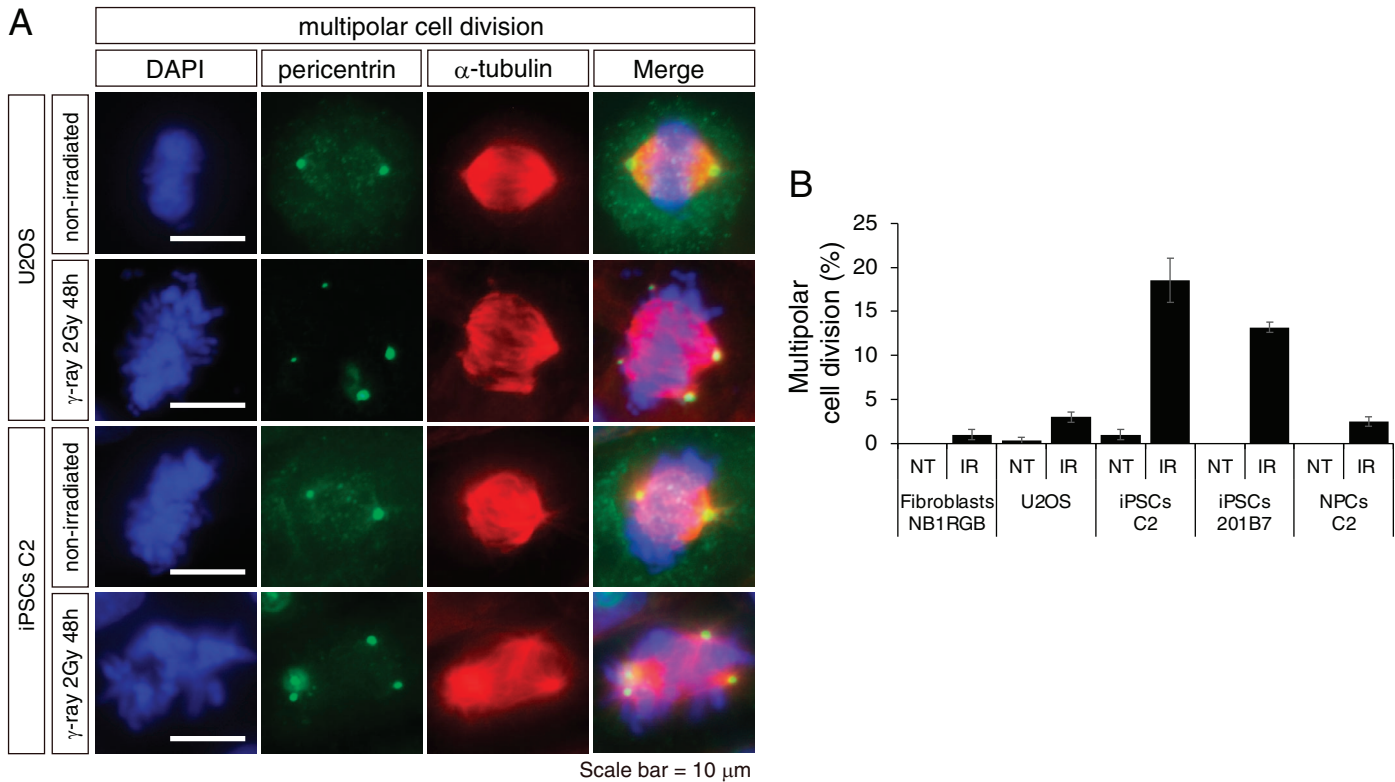


FIG. 2. Multipolar cell division in human induced pluripotent stem cells (hiPSCs) and differentiated cells after gamma ray exposure. Panel A: Cells (NB1RGB cells, U2OS cells, C2 iPSCs, 201B7 fibroblasts, and NPCs) were untreated or exposed to 2 Gy of gamma rays and incubated for 48 h. Images show mitotic cells. Pericentrin and α -tubulin antibodies were used as centrosome (green) and microtubule markers (red), respectively. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI: blue). Scale bars represent 10 μ m. Panel B: At least 200 multipolar cells were counted in mitotic cells. All experiments were independently performed at least three times. Error bars indicate standard error.

Radioprotective Effect of DMSO after Irradiation with Carbon Ions

Irradiation with heavy ions induces complicated DNA damage called clustered DNA damage. We previously reported the efficient induction of centrosome overduplication and

multipolar cell division after irradiation of mammalian somatic cells with heavy ions (23). Clustered DNA damage and mitotic catastrophe induced by heavy ions are expected to be efficient in causing tumor cell death during radiotherapy. We used a carbon-ion beam generated by the HIMAC at the

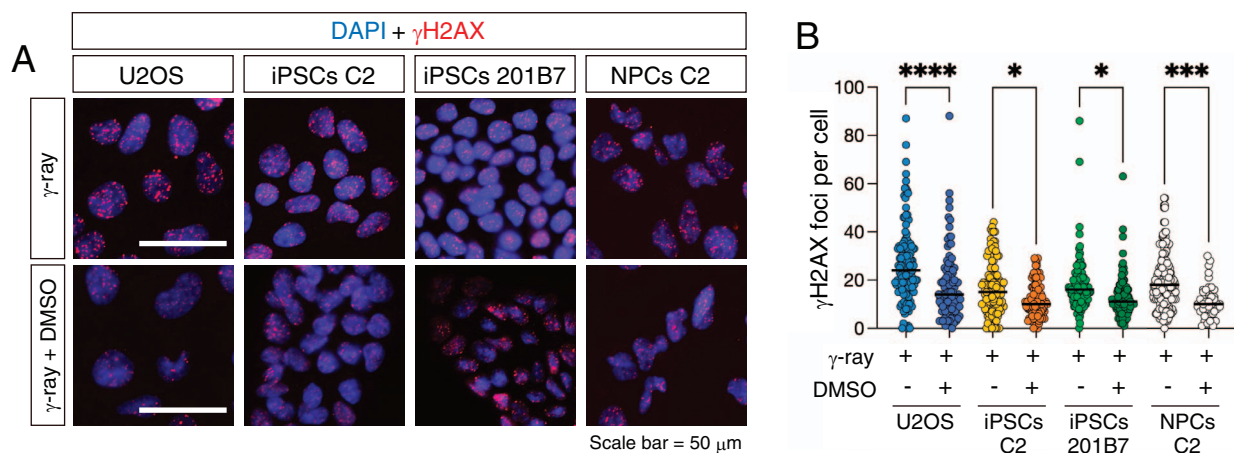


FIG. 3. Dimethyl sulfoxide (DMSO) attenuates DNA damage after gamma ray irradiation in human induced pluripotent stem cells (hiPSCs). Panel A: DMSO (0.1 M) was added 30 min before irradiation, and cells (NB1RGB cells, U2OS cells, C2 iPSCs, 201B7 fibroblasts, and NPCs) were exposed to 2 Gy of gamma rays. Cells were stained using DAPI and γ H2AX antibody 30 min after irradiation. Scale bars represent 50 μ m. Panel B: γ H2AX foci per nucleus were counted using cell profiler and graphed. At least 100 nuclei were counted. All experiments were independently performed at least three times. Data were analyzed using one-way analysis of variance and Tukey's post-hoc test. * $P < 0.1$ and **** $P < 0.0001$.

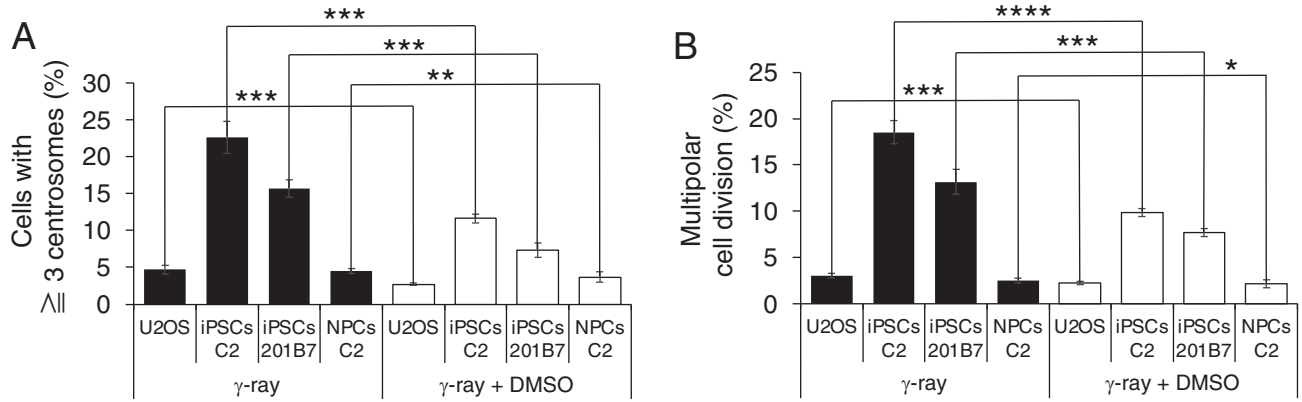


FIG. 4. Dimethyl sulfoxide (DMSO) attenuates centrosome aberrations after gamma ray irradiation in human induced pluripotent stem cells (hiPSCs). Panel A: DMSO (0.1 M) was added 30 min before irradiation, and cells (NB1RGB cells, U2OS cells, C2 iPSCs, 201B7 fibroblasts, and NPCs) were exposed to 2 Gy of gamma rays. Cells were stained using 4',6-diamidino-2-phenylindole (DAPI), pericentrin, and α -tubulin antibodies 48 h after irradiation. Cells with excess centrosomes were counted and graphed. Panel B: Multipolar cell division in 100 mitotic cells was counted and graphed. All experiments were performed at least three times independently. Data were analyzed using Student's *t*-test. **P* < 0.5, ***P* < 0.1, ****P* < 0.05, and *****P* < 0.01.

National Institute for Quantum Science and Technology to investigate radioprotective effects of DMSO against heavy ion in PSCs. The hiPSCs and differentiated cells were exposed to 2 Gy of carbon ions (13 keV/μm).

First, we addressed the effect of DMSO against DNA damage by measuring γ H2AX foci formation (Fig. 5A and B). DMSO was added 30 min before irradiation, and the cells were incubated for 30 min after exposure to carbon ions. Subsequently, the cells were fixed and stained with γ H2AX antibody. In the hiPSCs (C2) and differentiated cells (U2OS cells and NPCs), DMSO efficiently suppressed the generation of DNA damage. We then assessed centrosome overduplication and multipolar cell division

48 h incubation after irradiation with carbon ions (Fig. 6A and B). Carbon-ion irradiation efficiently induced centrosome overduplication (U2OS cells: 8.0%, iPSCs 30.5%, and NPCs: 6.2%) and multipolar cell division (U2OS cells: 6.5%, iPSCs: 24.5%, and NPCs: 3.3%) compared to gamma-ray irradiation in all cells. DMSO-treated cells suppressed centrosome overduplication (U2OS cells: 6.0%, iPSCs 18.5%, and NPCs: 4.8%) and multipolar cell division (U2OS cells: 4.0%, iPSCs 14.0%, and NPCs: 2.2%). These results indicate that DMSO exhibits a radioprotective effect against DNA damage and centrosome aberrations in cells irradiated with gamma rays and carbon ions.

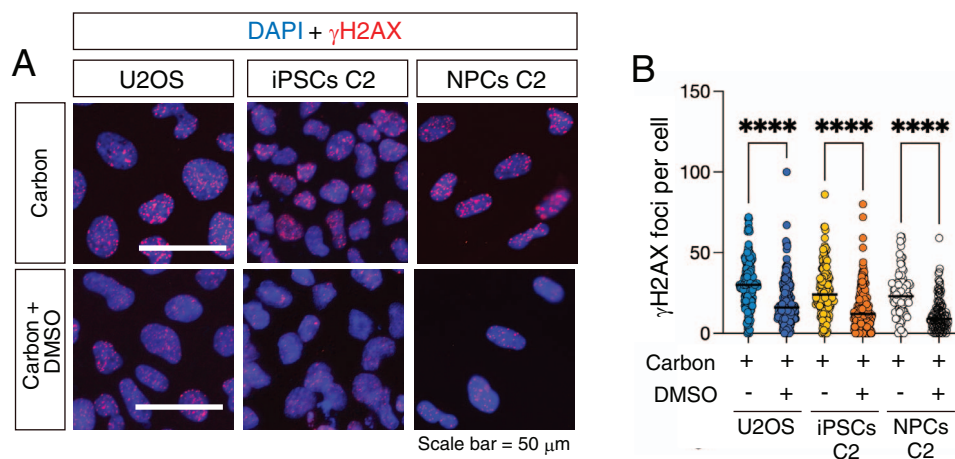


FIG. 5. Dimethyl sulfoxide (DMSO) attenuates DNA damage after carbon ion irradiation in human induced pluripotent stem cells (hiPSCs). Panel A: DMSO (0.1 M) was added 30 min before irradiation, and cells (NB1RGB cells, U2OS cells, C2 iPSCs, 201B7 fibroblasts, and NPCs) were exposed to 2 Gy of carbon ions. Cells were stained using 4',6-diamidino-2-phenylindole (DAPI) and γ H2AX antibody 30 min after irradiation. Scale bars represent 50 μm. Panel B: γ H2AX foci per nucleus were counted using cell profiler and graphed. At least 100 nuclei were counted. All experiments were independently performed at least three times. Data were analyzed using one-way analysis of variance and Tukey's post-hoc test. *****P* < 0.0001.

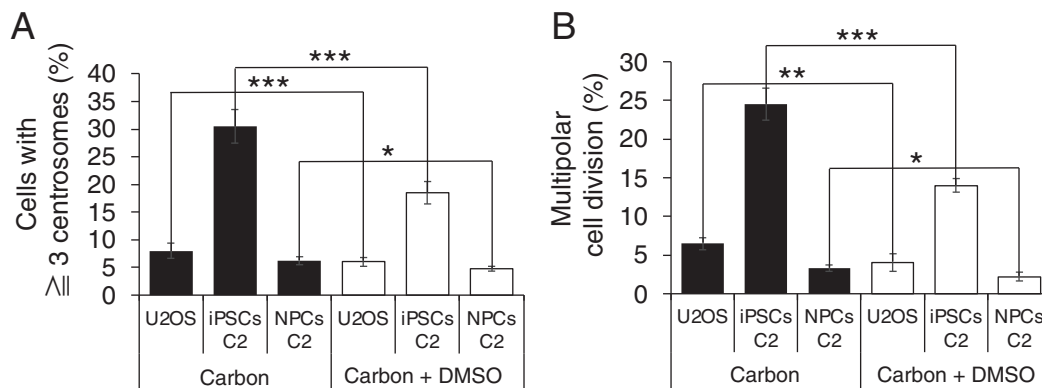


FIG. 6. Dimethyl sulfoxide (DMSO) attenuates centrosome aberrations after carbon ion irradiation in human induced pluripotent stem cells (hiPSCs). Panel A: DMSO (0.1 M) was added 30 min before irradiation, and cells (NB1RGB cells, U2OS cells, C2 iPSCs, 201B7 fibroblasts, and NPCs) were exposed to 2 Gy of carbon ions. Cells were stained using 4',6-diamidino-2-phenylindole (DAPI), pericentrin, and α -tubulin antibodies 48 h after irradiation. Cells with excess centrosomes were counted and graphed; Panel B: Multipolar cell division in 100 mitotic cells was counted and graphed. All experiments were performed at least three times independently. Data were analyzed using Student's t-test. * $P < 0.5$, ** $P < 0.1$, and *** $P < 0.05$.

DISCUSSION

In this study, we demonstrated that radiation exposure efficiently induces centrosome overduplication in hiPSCs. This enhanced susceptibility to centrosome overduplication in hiPSCs could be attributed to their unique cell cycle dynamics, characterized by a short G_1 phase and suppressed cell cycle checkpoints (28). Due to their short G_1 phase, PSCs exhibit rapid cell cycle progression (approximately 14–16 h) compared to somatic cells (approximately 24 h) (28). We allowed a 48-h incubation period after radiation exposure to allow for centrosome duplication. Considering that hiPSCs require approximately 1.5 times the cell cycle period of the somatic cells within this incubation timeframe, it permits approximately 1.5 times the centrosome duplication cycle (28). Additionally, PSCs exhibit high expression levels of various CDKs, including CDK2/4, coupled with suppressed expression of the CDK2 inhibitor p21 (28). Given that the centrosome duplication cycle is regulated by CDK2 activity, its heightened expression in PSCs may accelerate this process.

In contrast, the radical scavenger DMSO mitigates radiation-induced DNA damage by protecting DNA against free radicals, particularly OH radicals. Protective effects of DMSO extend across a broad spectrum of LET (24). Despite a decrease in indirect action of OH radicals with increasing LET, they persist in the high-LET radiation range. Multiple studies indicate that this indirect action contributes to cell death after high LET irradiation, and DMSO exhibits radioprotective effects even for densely ionizing radiation (24, 25, 32, 33).

DMSO also suppressed centrosome overduplication, which is a reasonable outcome considering that radiation induces cell cycle checkpoint activation, leading to centrosome overduplication. The reduction in DNA damage from DMSO treatment

likely contributes to the suppression of centrosome overduplication. Additionally, DMSO exhibited a radioprotective effect in hiPSCs, which are prone to accumulating spontaneous DNA damage due to DNA replication stress and high energy metabolism. The combination of endogenous genome stress and radiation exposure can lead to cell death and heightened radiosensitivity. Protective effects of DMSO extend not only to induced DNA damage, such as from radiation exposure, but also to spontaneous DNA damage caused by free radicals. Although high doses of DMSO treatment (0.4 M) induce cytotoxicity in hiPSCs, lower concentrations (0.1 M) of DMSO may contribute to genome maintenance in hiPSCs.

In summary, our study demonstrates that gamma-ray and carbon-ion radiation induce markedly increased centrosome overduplication and multipolar cell division in hiPSCs. Additionally, the radical scavenger DMSO effectively suppressed DNA damage and centrosome aberrations in hiPSCs. These findings are valuable for ensuring the quality control of PSCs and advancing regenerative medicine. Notably, the Japan Aerospace Exploration Agency (JAXA) has plans to culture tissue organoids from PSCs in space stations because the microgravity situation offers an advantage for efficient organoid culture. However, cells are exposed to galactic radiation that includes protons and heavy ions. Therefore, efficient radioprotection methods are crucial. Our results contribute to the development of radiation protection strategies for regenerative medicine and space medicine.

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