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Biology Contribution

Dimethyl Sulfoxide Prevents Radiation-Induced Oral Mucositis Through Facilitating DNA Double-Strand Break Repair in Epithelial Stem Cells

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Summary

We show that dimethyl sulfoxide (DMSO) exhibits marked protective activity against radiation-induced oral mucositis in mice without tumor protection, and the efficacy of DMSO is superior to that of recombinant human keratinocyte growth factor and amifostine. Administration of DMSO significantly prevents the loss of proliferative lingual epithelial stem and

Purpose: Oral mucositis is one of the most prevalent side effects in patients undergoing radiation therapy for head and neck cancers. Current therapeutic agents such as palifermin recombinant human keratinocyte growth factor and amifostine do not efficiently or fully prevent mucositis. Dimethyl sulfoxide (DMSO), a free-radical scavenger, has shown therapeutic benefits in many preclinical and clinical studies. This study aimed to investigate the efficacy of DMSO in a clinically relevant mouse model of acute, radiation-induced oral mucositis.

Methods and Materials: Oral mucositis was induced by a high single and fractioned irradiation of the head and neck area in C57BL/6J mice, and the effects of DMSO (by intraperitoneal injection) were assessed by macroscopic and histopathological examination. Epithelial stem and progenitor cells were analyzed by immunohistochemical staining of p63 and Ki-67, and DNA double-strand breaks (DSBs) were visualized by immunofluorescence detection of γ-H2AX. Tumor xenograft was obtained using CAL-27 cells.

Results: Pretreatment with DMSO protected the oral mucosa from severe acute radiation injury, reduced the extent of radiation-induced weight loss, and had no significant effects on tumor weight in irradiated or nonirradiated xenograft mice. Furthermore, the efficacy of

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progenitor cells upon irradiation by facilitating DNA double-strand break repair. The high efficacy and low toxicity of DMSO suggest possible therapeutic application.

DMSO was superior to that of recombinant human keratinocyte growth factor and amifostine. DMSO treatment prevented the loss of proliferative lingual epithelial stem and progenitor cells upon irradiation. More interestingly, the average levels of γ-H2AX foci were significantly decreased in p63-positive epithelial stem cells at 6 hours, but not at 2 hours, after irradiation, indicating that DMSO facilitated DNA DSB repair rather than suppressing the indirect action of irradiation.

Conclusions: DMSO prevents the loss of proliferative lingual epithelial stem and progenitor cells upon irradiation by facilitating DNA DSB repair, thereby protecting against radiation-induced mucositis without tumor protection. Given its high efficacy and low toxicity, DMSO could be a potential treatment option to prevent radiation-induced oral mucositis.

Introduction

Radiation therapy is an important treatment modality for patients with head and neck cancers. Although ongoing technical advances allow a more targeted delivery of higher doses of radiation to the cancer cells, indirect damage to the normal tissues near the target inevitably leads to a common and frequently debilitating side effect (1). It has been reported that all patients undergoing radiation therapy for treatment of head and neck cancers develop acute oral mucositis, a condition characterized by the complete breakdown of the mucosal epithelia and the presence of ulcerative lesions that involves pain and swallowing difficulties, resulting in the restriction of oral intake, dehydration, and weight loss (2). Furthermore, oral mucositis frequently enhances the risk of local and systemic infections, increases the length of hospitalization, and leads to impaired quality of life in affected individuals (3). Consequently, oral mucositis may lead to delayed treatment and dose reductions, thus compromising local tumor control and overall patient survival (4).

Several types of therapy, such as local anesthetics, anti-inflammatory agents, and antiulcer agents, have been used for the prevention or treatment of radiation-induced oral mucositis; however, the clinical response elicited by these treatments is poor (5-8). Palifermin (recombinant human keratinocyte growth factor [rhKGF]) has proved its efficacy in reducing the severity and duration of mucositis in preclinical models and patients. However, its application is limited to patients undergoing myeloablative conditioning before hematopoietic stem cell transplantation because keratinocyte growth factor was shown to enhance the growth of human epithelial tumor cells in vitro and in vivo (9). Amifostine, a free-radical scavenger that provides significant tissue protection when administrated intravenously before irradiation, has been approved by the US Food and Drug Administration as a drug for inhibiting xerostomia (10). Its use is also limited by debilitating side effects, including nausea/vomiting and hypotension (6). The poor efficacy of current approaches makes identification of novel molecular targets to develop safer and more effective radioprotective agents against oral mucositis an urgent need.

The pathogenesis and progression of oral mucositis is a complex process that has been partially described at the molecular level. In the initiation stage of radiation-induced oral mucositis, the generation of reactive oxygen species (ROS) and subsequent DNA damage activate several signaling pathways in the submucosa and epithelium, which in turn lead to atrophy, loss of barrier function, and ulceration (11, 12). Using either radical scavengers or superoxide dismutase mimetics, many studies have recently demonstrated that ROS are an important early trigger resulting in oral mucositis (13, 14). Dimethyl sulfoxide (DMSO) is an organosulfur compound that is frequently used as a vehicle for drug therapy. Because of its ROS scavenging activities and anti-inflammatory properties, DMSO exhibits therapeutic benefits in the treatment of inflammatory and gastrointestinal diseases, chronic pain, and cardiac and central nervous system damage (15-17). In this study, we explored whether DMSO could protect mice from mucositis induced by a high single dose and clinically relevant fractioned doses of head-only irradiation.

Methods and Materials

Mice

Six- to 8-week-old male SPF C57BL/6J mice (22-25 g) and male BALB/C nude mice (22-24 g) used in the study all were purchased from Beijing HFK Bioscience Co. Ltd. The animals were housed in the Laboratory Animal Center and allowed to have free access to pellet food and water. All described experiments strictly adhered to the Laboratory Animals Guideline of welfare and ethics (GB/T 35892-2018).

Radiation-induced oral mucositis

Mice were anesthetized with ketamine during irradiation. The technique and setup for head-only radiation treatment in mice was modified based on previously published studies (7, 18). The head and neck areas were irradiated by guiding each animal into a customized box in a supine position. Within the box, their bodies, but not their heads, were

shielded with custom-made 6-mm lead. Ionizing radiation was delivered with the RS2000 biological x-ray irradiator (160 KVp, 25 mA, Rad Source) with a dose rate of 1.325 Gy/min. The dose rate of radiation field was determined by a 0.6 cm^3 FAMER ion chamber connected with a PTW UNIDOS dosimeter during the radiation procedure and was constant. Therefore, the single-fraction dose was achieved by adjustment of the irradiation time.

After radiation, mice were allowed to recover on a heated pad and were housed in a climate- and light-controlled environment with 4 mice per cage. They were allowed free access to water and soft, nutritive food, such as eggs. For the tumorigenicity assay, mice received a dose of 12 Gy, whereas for other experiments, mice received single-dose irradiation of 16.5 Gy or fractionated irradiation of 8 Gy × 3 (8 Gy/d for 3 days). Mice survival was recorded daily, and body weight was recorded every second day. Mice that experienced weight loss >10 g and limited motility were euthanized by anesthesia. For morphologic analyses, mice were sacrificed by dislocating cervical vertebrae at the appropriate time points for each experiment.

DMSO administration in mice

DMSO administration in mice is described in the Supplementary Methods (available online at https://doi.org/10.1016/j.ijrobp.2018.07.2010).

Evaluation of oral mucositis

Mice were humanely killed, and the whole tongue was then removed from the oral cavity. To analyze radiation-induced oral mucositis macroscopically, some tongues were stained in a solution of 1% toluidine blue (TB). Repeated wiping with an acetic acid—soaked swab was continued until there was no further recovery of dye. Light or no dye uptake indicated a negative result, whereas a deep, royal blue staining in epithelium defects indicated a positive result (19).

After 1% TB staining, the tongues were dissected longitudinally in the median plane (dividing the ulcer into identical halves), kept in 10% buffered formalin, then embedded in paraffin; 5-μm sections were stained with hematoxylin and eosin (H&E). Tongue histology was scanned at 40× magnification using a Leica DM4000B digital microscope equipped with image-capturing software. Histometric analysis, including ulcer size and mucosal thickness, was determined using Leica LAS-X software. At least 10 independent measurements from 4 different tongue sections were made for each study. The scorer was blinded and was not aware of the condition of the specimens.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed as described in the Supplementary Methods (available online at https://doi.org/10.1016/j.ijrobp.2018.07.2010).

Tumorigenicity in vivo

Tumorigenicity in vivo was performed as described in the Supplementary Methods (available online at https://doi.org/10.1016/j.ijrobp.2018.07.2010).

Statistical analysis

Survival was analyzed by Kaplan-Meier analysis with the log-rank test. Other analyses were performed in triplicate or greater, and the means obtained were used for independent *t* tests. Statistical analyses were carried out using the Graph Pad Prism 6. Statistical significance was defined as $P < .05$. Data presented here represent a minimum of 2 or 3 experiments and, where appropriate, data are expressed as means ± SD.

Results

DMSO administration protects mice against ulcerative oral mucositis induced by single, high-dose, local irradiation of the head and neck

The radioprotective effects of DMSO against radiation-induced ulcerative oral mucositis were first studied using the single-dose irradiation mouse model that recapitulates the features of severe clinical oral mucositis (20). In preliminary studies, we determined that a single 16.5-Gy dose of x-ray reliably resulted in overt tongue mucosal ulceration (data not shown). For oral mucositis prevention, a single dose of DMSO at 6 g/kg was administered intraperitoneally (i.p.) to the mice, starting 1 hour before radiation. As shown in Figures 1A and 1B, exposure to a single 16.5-Gy dose of radiation brought about a total loss of more than 45% of initial body weight because of a dramatic reduction in food and water consumption, leading to mortality of all irradiated mice within 15 days. In contrast, mice pretreated with DMSO showed less significant weight loss and gained weight up to normal ranges by day 20. No mice treated with DMSO died during the experimental period.

To determine the protective effect of DMSO against the mucosal ulceration, mouse tongues were removed and stained with TB. As shown in Figure 1C, ulcers, indicated by deep blue staining with TB, started to appear at day 7 and reached maximum severity at days 9 to 11 after radiation. Ulcers were always localized at the posterior dorsal surface of the tongue. Surprisingly, DMSO administration just before radiation almost completely prevented the appearance of ulcers in irradiated mice. To further assess the extent of ulceration, tongue sections were stained with H&E and processed for analysis to examine the ulcer size and thickness of the remaining epithelium. As showed in Figure 1D, oral mucositis, characterized by thinning epithelium, flattened tongue papillae, and surface ulceration along with inflammatory cells infiltration in the adjacent

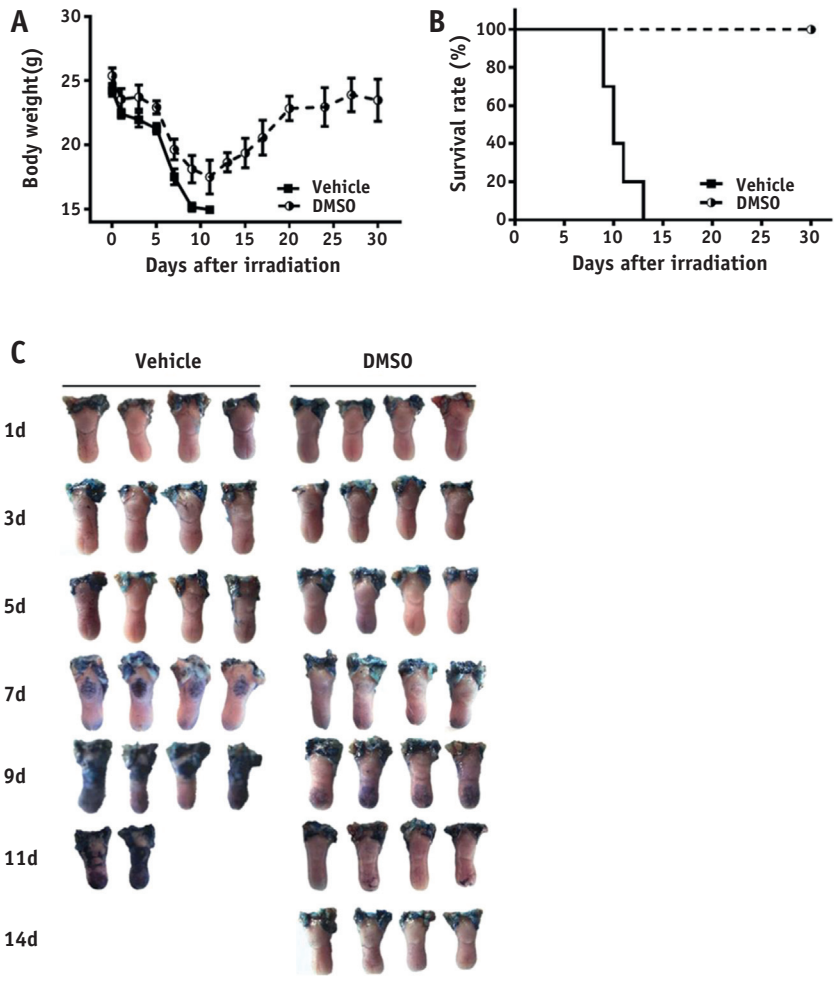

Fig. 1. DMSO prevents radiation-induced oral mucositis in mice. (A) Mouse body weight changes and (B) Kaplan-Meier survival rate curve of 2 groups (n = 7) administered saline (vehicle) and DMSO (6 g/kg) 1 hour before 16.5-Gy local irradiation to the head. Results representative of 2 analogous experiments are presented. (C) Toluidine blue and (D) hematoxylin–eosin staining of mouse tongues obtained at the indicated time points (n = 4 or 2 because of death from day 10) after 16.5-Gy local irradiation to the head. Mice were treated with saline (vehicle) or DMSO (6 g/kg) 1 hour before irradiation. The vertical lines in the images of tongues from vehicle mice highlight the ulcer boundary, and dotted lines in all images indicate the epithelial-stromal boundary. Scale bar, 50 μm. (E) Quantification of lingual ulcer size and (F) mucosal thickness in (D). Results representative of 3 analogous experiments are presented. Data represent mean ± SD. $^{**}P < .01$, $^{***}P < .001$ against vehicle-treated mice. *Abbreviations:* DMSO = dimethyl sulfoxide; non-IR = nonirradiated.

connective tissue, was evident at day 5. It became severe at days 9 to 11 after radiation in all animals treated with vehicle and was all resolved in mice treated with DMSO, as seen by the preservation of the epithelial layer, increase of mucosal thickness, and reduction of inflammatory infiltrates and edema. Morphometric analysis of H&E-stained dorsal tongue sections confirmed the protective effect of DMSO against oral mucositis induced by a single high dose of local irradiation to the head and neck of mice (Figs. 1E and 1F). A similar protective effect of DMSO was also seen in the tip and ventral dorsal tongue mucosa of irradiated mice (Figs. E1A-E1D; available at https://doi.org/10.1016/j.ijrobp.2018.07.2010). These results show that DMSO is an effective radioprotective agent in reducing irradiation-induced tongue mucosal injury in mice.

Effects of DMSO administration protocols on radiation-induced ulcerative oral mucositis in mice

To determine the dose required to achieve the radioprotective effects, mice were administered a single dose of DMSO (i.p.) at 4 g/kg and 6 g/kg, starting 1 hour before radiation. As shown in Figures E2A and E2B (available at https://doi.org/10.1016/j.ijrobp.2018.07.2010), both groups pretreated with DMSO at 4 g/kg and 6 g/kg showed less significant weight loss compared with irradiated mice receiving vehicle; gained weight up to normal ranges by day 20 and day 30, respectively; and showed 100% survival from a single 16.5-Gy dose local irradiation to the head and neck. Extensive ulceration, evidenced by deep blue staining with TB and morphometric analysis of H&E stained dorsal

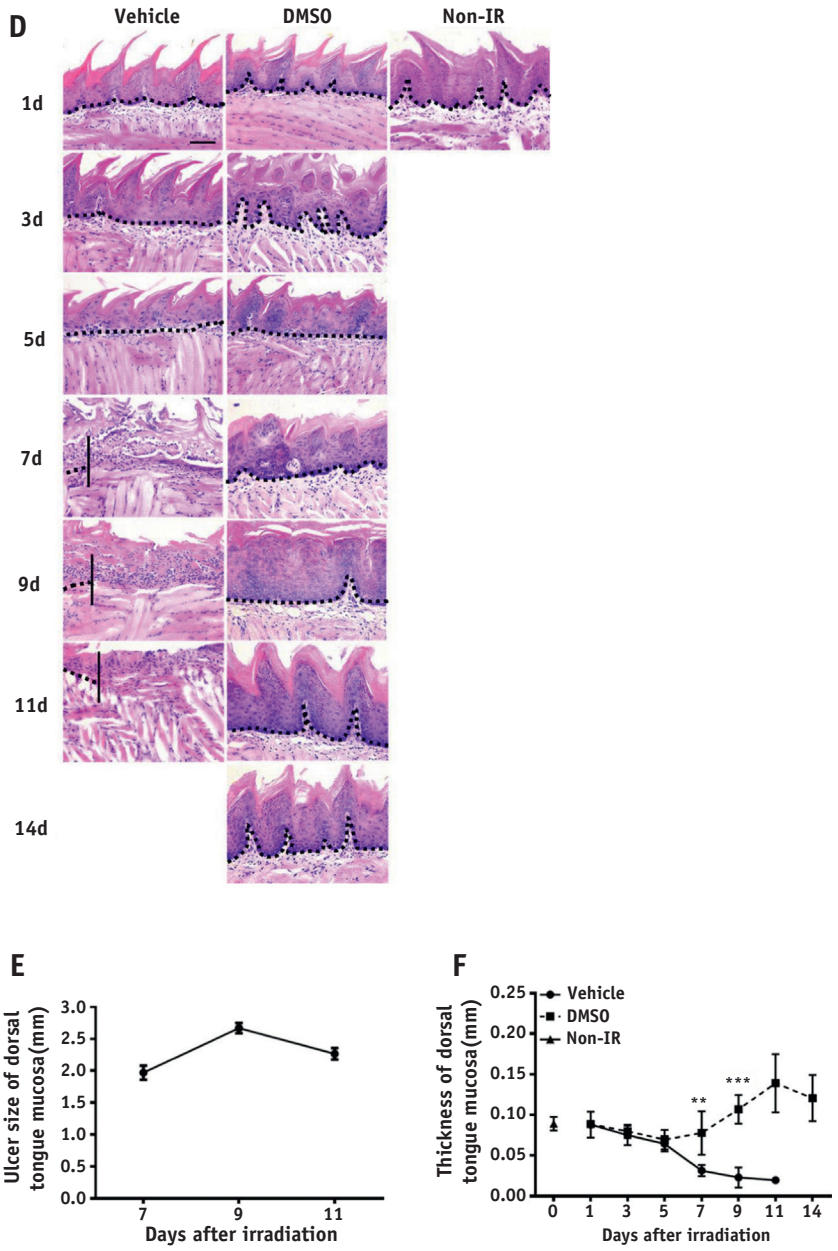

Fig. 1. (Continued)

tongue sections, was seen in all animals treated with vehicle but was absent in 87.5% of mice treated with DMSO at 4 g/kg and in 100% of mice treated at 6 g/kg (Figs. E2C and E2D; available at https://doi.org/10.1016/j.ijrobp.2018.07.2010). In addition to the prevention of overt tongue ulceration, administration of DMSO also significantly increased the mucosal thickness in nonulcerated areas of mouse tongue (Fig. E2E; available at https://doi.org/10.1016/j.ijrobp.2018.07.2010).

To investigate the optimal timing of DMSO administration for prevention of oral mucositis, mice were injected with DMSO (6 g/kg, i.p.) at 6 hours, 3 hours, and 1 hour before irradiation. A single-fraction 16.5-Gy head and neck irradiation resulted in 100% tongue ulceration with completely denuded mucosal epithelium in vehicle control mice. However, administration of DMSO significantly reduced the incidence of tongue ulceration in a time-dependent fashion to 12.5% (1 of 8 mice, $P < .05$), 12.5% (1 of 8 mice, $P < .05$), and 0% (0 of 8 mice, $P < .01$) at 6 hours, 3 hours, and 1 hour before irradiation, respectively (Fig. 2A). Quantitative analysis of ulcer size and epithelial thickness from H&E-stained dorsal tongue sections confirmed the protective effect of DMSO against radiation-induced ulcerative oral mucositis in mice (Figs. 2B and 2C). However, administration of DMSO after radiation showed no radioprotective effect compared with vehicle controls (Figs. E3A-E3C; available at https://doi.org/10.1016/j.ijrobp.2018.07.2010).

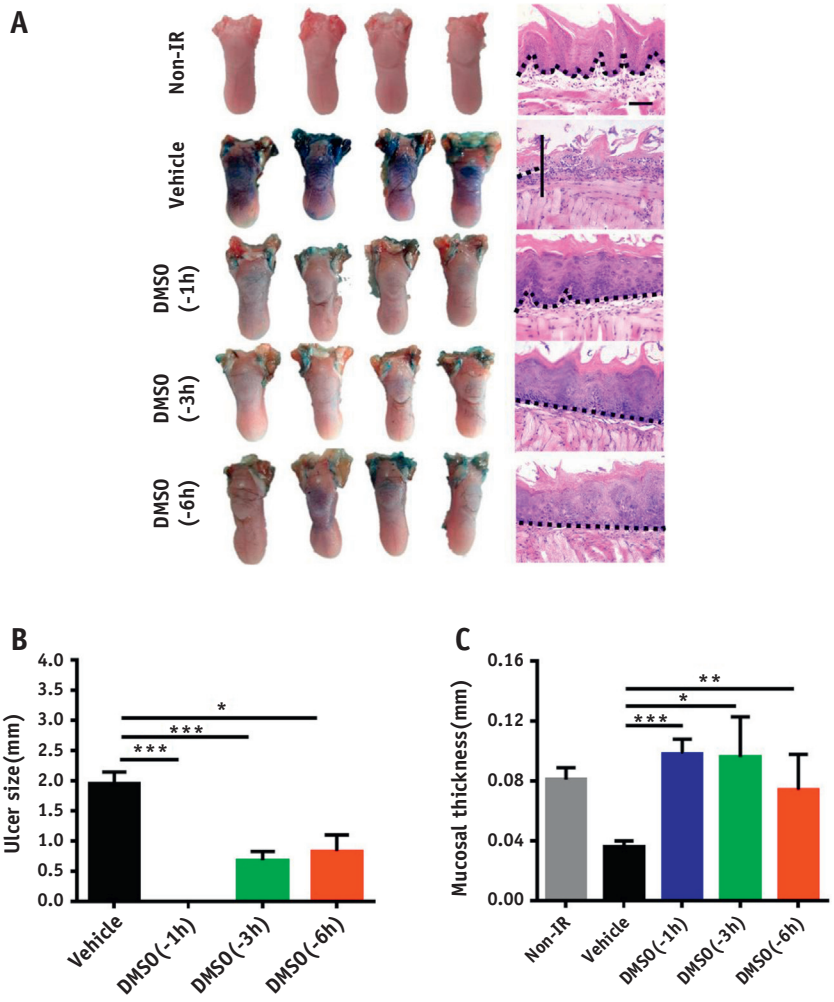

Fig. 2. The optimal timing regimen of DMSO for radioprotection against radiation-induced oral mucositis. (A) Toluidine blue and hematoxylin–eosin staining of mouse tongues obtained 7 days after 16.5-Gy local irradiation to the head. Mice were treated with saline (vehicle) or DMSO (6 g/kg) at 1 hour, 3 hours, and 6 hours before irradiation (n = 4). The vertical lines in the images of tongues from vehicle mice highlight the ulcer boundary, and dotted lines in all images indicate the epithelial-stromal boundary. Scale bar, 50 μm. (B) Quantification of lingual ulcer size and (C) mucosal thickness in (A). Results representative of 3 analogous experiments are presented. Data represent mean ± SD. $*P < .05$, $**P < .01$, $***P < .001$ against vehicle-treated mice. *Abbreviations:* DMSO = dimethyl sulfoxide; non-IR = nonirradiated.

Palifermin (rhKGF) and amifostine (WR2721) have demonstrated their efficacy in reducing mucositis in preclinical models and patients (6). We therefore compared the effect of DMSO with the known radioprotectors against radiation-induced mucositis after 16.5-Gy irradiation of the head and neck in mice. Animals received rhKGF and WR2721 treatment with a clinical regimen: rhKGF (6.25 mg/kg, i.p.) administered daily for 3 days before irradiation and daily for 3 days 24 hours after radiation (7) and WR2721 (200 mg/kg, i.p.) injected at a single dose 30 minutes before radiation. As shown in Figure 3A, all treated groups showed less significant weight loss compared with irradiated mice receiving vehicle; mice gained weight up to normal ranges by day 17, day 23, and day 23 in the DMSO (6 g/kg, −1 hour, i.p.), rhKGF, and WR2721 treatment groups, respectively. Two mice (out of 7) died in both the rhKGF and WR2721 treatment groups during the experimental period, whereas no mice died in the DMSO-treated groups (Fig. 3B). On the basis of the quantitative analysis of ulcer size and epithelial thickness from H&E-stained dorsal tongue sections, DMSO, rhKGF, and WR2721 all provided significant protection from radiation-induced mucositis, although small ulcers were still observed in rhKGF- and WR2721-treated mice (Figs. 3C-3E). These results indicated that DMSO may be a superior radioprotector compared with palifermin and amifostine in preventing mucositis in patients.

Because clinical practice of radiation therapy uses fractioned doses of local radiation (4, 5, 7), we next applied cumulative local x-ray doses to 24 Gy of x-ray radiation to the head and neck area of mice as 3 fractions of 8 Gy. DMSO (4 g/kg) or vehicle was administered to

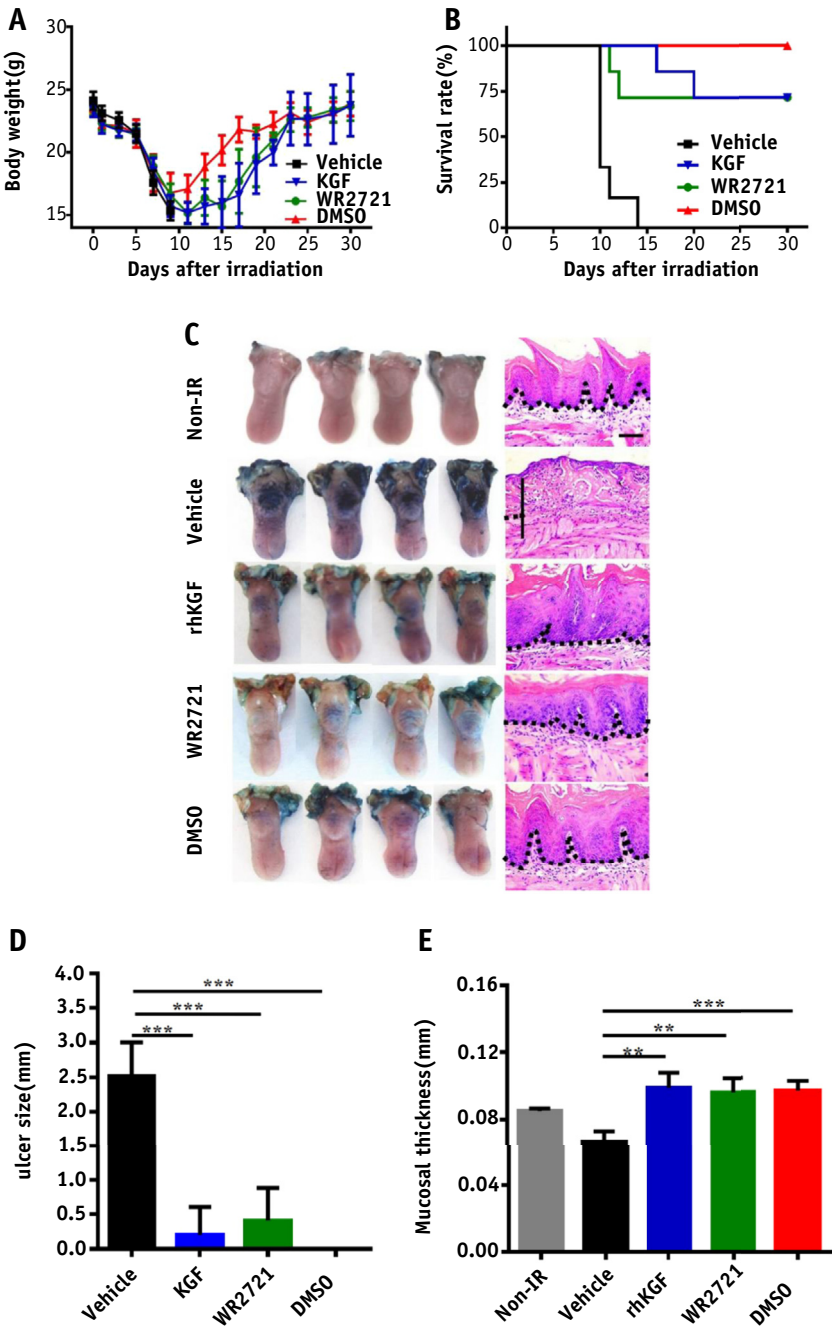

Fig. 3. The efficacy of DMSO in preventing radiation-induced oral mucositis in mice is superior to that of amifostine or rhKGF. (A) Mouse body weight changes and (B) Kaplan-Meier survival rate curve of 4 groups (n = 7) administered with saline (vehicle), WR2721 (200 mg/kg, −30 minutes), rhKGF (6.25 mg/kg, daily for 3 days before irradiation and then daily for 3 days beginning 24 hours after radiation) and DMSO (6 g/kg, −1 hour) before or after 16.5 Gy local irradiation to the head. Results representative of 2 analogous experiments are presented. (C) Toluidine blue and hematoxylin−eosin staining of mouse tongues obtained 7 days after 16.5-Gy local irradiation to the head. Mice were treated with saline, WR2721, rhKGF, and DMSO as indicated before or after irradiation (n = 4). The vertical lines in the images of tongues from vehicle mice highlight the ulcer boundary, and dotted lines in all images indicate the epithelial-stromal boundary. Scale bar, 50 μm. (D) Quantification of lingual ulcer size and (E) mucosal thickness in (C). Results representative of at least 2 analogous experiments are presented. Data represent the mean ± SD. $^{**}P < .01$, $^{***}P < .001$ against vehicle-treated mice. *Abbreviations:* DMSO = dimethyl sulfoxide; KGF = keratinocyte growth factor; non-IR = nonirradiated; rhKGF = recombinant human keratinocyte growth factor.

the designated mice at 1 hour before each irradiation dose. Seven days after starting irradiation, lingual ulcers were found in 100% of the tongues in mice that received the 3 × 8 Gy dose. DMSO administration provided complete protection from radiation-induced mucositis, as measured by both ulcer size and epithelial thickness (Figs. E6A-E6C; available at https://doi.org/10.1016/j.ijrobp.2018.07.2010). These studies demonstrated that the protection conferred by DMSO may be effective under the conditions of single doses as well as clinically relevant fractioned dosage of local irradiation.

DMSO prevents the loss of proliferative capacity of the lingual epithelial stem and progenitor cells after irradiation in mice by facilitating DNA DSBs repair

As previously reported, the specific stem cells in the lingual epithelium facilitate the formation of stratified epithelia and regenerate injured epithelial tissue (21). This outcome prompted us to analyze the expression of p63 in the oral mucosa of mice exposed to radiation and treated with DMSO or vehicle. A well-established marker of epithelial stem cells, p63 is abundantly expressed in the basal layers of the normal oral mucosa. As shown in Figures 4A and 4C, the number of p63-positive cells in the dorsal tongue mucosa of irradiated mice treated with vehicle started to decrease at day 1, reached a minimum at day 7, and was lowest from day 9 to day 11 after radiation. In contrast, animals pretreated with DMSO (6 g/kg) showed a less significant decrease in the number of p63-positive cells compared with vehicle control mice, began recovery at day 7, and gained numberup to the normal ranges by day 9. A similar protective effect of DMSO was also seen in the tip and ventral tongue mucosa of irradiated mice (Figs. E4A-E4D; available at https://doi.org/10.1016/j.ijrobp.2018.07.2010). These results show that DMSO treatment prevents the loss of the epithelial stem and progenitor cell compartment after radiation.

The proliferative capacity of the epithelial stem and progenitor cells is essential for tissue homeostasis and repair. By using the proliferation marker Ki-67, we found that Ki-67 was expressed only in the bottommost layer of the normal mouse tongue mucosa, indicating that most of the p63-positive stem cells were actually cycling (Fig. 4B). Consistent with the loss of the p63-positive stem cells, the Ki-67−positive proliferating cells were significantly decreased in number in the dorsal tongue mucosa of mice after irradiation, whereas pretreatment with DMSO significantly prevented the decrease and promoted recovery of Ki-67−positive proliferating cells (Fig. 4D). A similar protective effect of DMSO was also seen in the tip and ventral tongue mucosa of irradiated mice (Figs. E5A-E5D; available at https://doi.org/10.1016/j.ijrobp.2018.07.2010). Taken together, our results indicate that DMSO administration prevents the loss of proliferative capacity of the epithelial stem and progenitor cells after radiation, which in turn contributes to protection against radiation-induced tongue mucosal injury in mice.

To examine the potential role of DNA damage and repair in DMSO-enhanced group the epithelial stem cells regeneration after radiation, we detected DNA DSBs by double-staining of γ-H2AX with p63. γH2AX ionizing radiation−induced repair foci are now considered indicative of DSB number, whereas kinetics of ionizing radiation−induced repair resolution serve as a surrogate for DSB repair (22). As shown in Figures 5A and 5B, the number of γH2AX foci in p63-positive cells was similar between the DMSO and vehicle control groups at the 2-hour point after irradiation, suggesting that the radioprotective effects of DMSO were not attributable to the suppression of the initial DSBs induced by ionizing radiation. However, 6 hours after irradiation, the average levels of γ-H2AX foci in p63-positive cells were significantly lower in the DMSO-treated group than in vehicle control groups. This result indicated that DMSO protected against epithelial stem-cell depletion through the facilitation of DNA DSB repair rather than through the suppression of DNA DSBs induced by irradiation.

Absence of tumor protection from irradiation by DMSO in vivo

To investigate whether the protective effect of DMSO against radiation-induced oral mucositis is specific for normal tissues, we used human-tongue squamous cell carcinoma CAL-27 grown subcutaneously in the neck area in nude mice (7). Xenografted mice were injected with DMSO (6 g/kg, i.p.) 1 hour before 12 Gy of local x-ray radiation, and the tumor volume was measured every 3 days. As shown in Figure 6A, the trend of tumor growth was notably suppressed in 2 irradiated groups, whereas DMSO administration showed no effect on the growth of the tumor. On day 30 after irradiation, mice were euthanized and tumor weights were measured. As shown in Figures 6B and 6C, the mean tumor weight was significantly decreased in 2 irradiated groups. In contrast, DMSO had no significant effects on tumor weight in irradiated or nonirradiated groups compared with placebo controls. In addition, we measured animal weights throughout the study and found that the weights were similar between 2 nonirradiated groups treated with vehicle or DMSO. As consistent with previous results, xenografted mice exposed to a single 12-Gy local radiation showed significant weight loss after irradiation, leading to death of 1 mouse at day 11. In contrast, mice pretreated with DMSO showed less significant weight loss, and no mice died during the experimental period (Fig. 6D). Therefore, DMSO was shown to prevent radiation-induced oral mucositis without tumor protection.

Discussion

Oral mucositis is the most severe side effect in patients receiving conventional radiation therapy for head and neck cancers. Currently used drugs, such as palifermin and

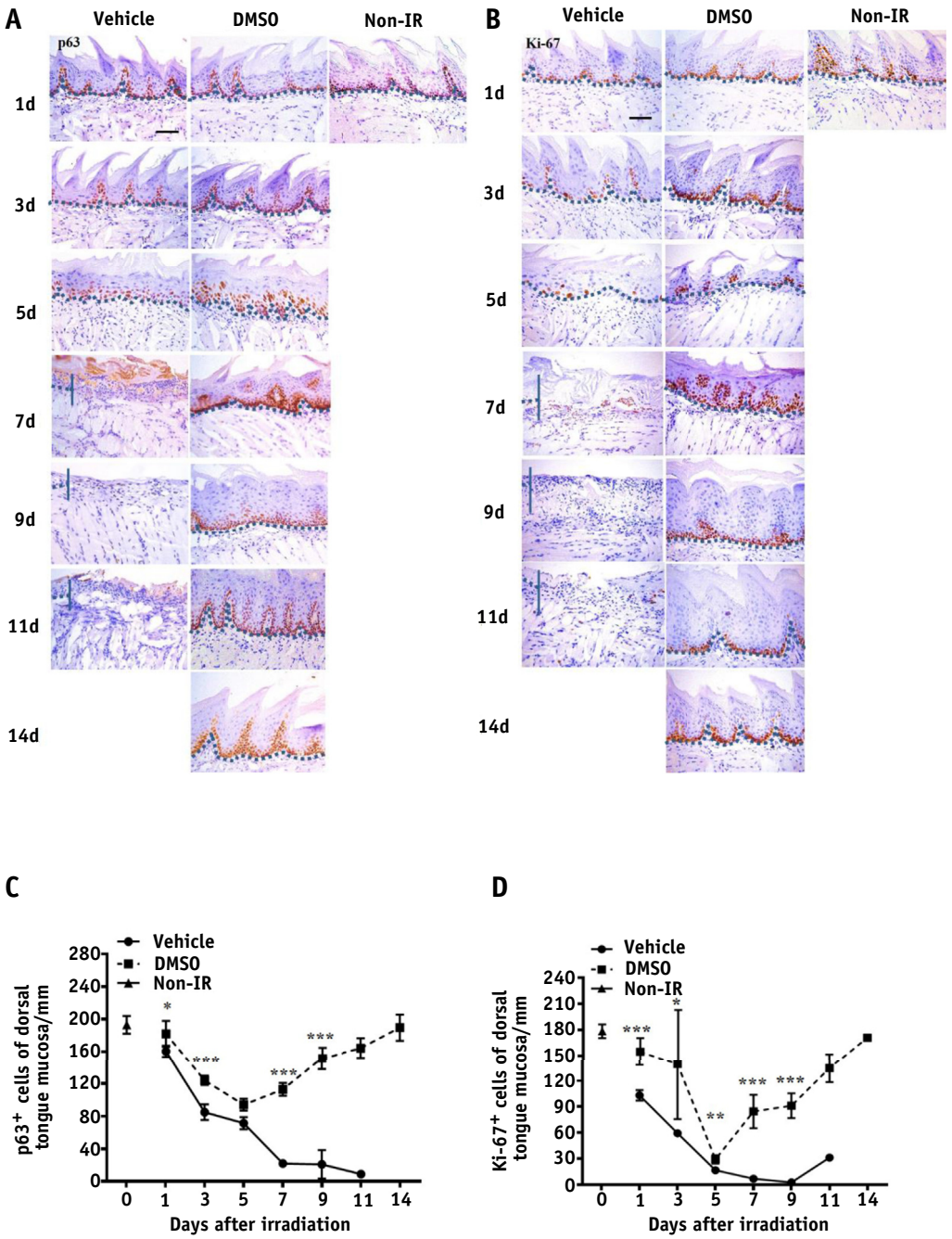

Fig. 4. DMSO accelerates regeneration of lingual epithelial tissue at the indicated time points after irradiation-induced injury. Immunohistochemistry of (A) the stem cell marker p63 and (B) the proliferation marker Ki-67 assay in dorsal mucosa of mouse tongue obtained at the indicated time points (n = 4 or 2 because of death on day 10) after 16.5-Gy local irradiation to the head. Mice were treated with saline (vehicle) or DMSO (6 g/kg) 1 hour before irradiation. The vertical lines in the images of tongues from vehicle mice highlight the ulcer boundary, and dotted lines in all images indicate the epithelial-stromal boundary. Scale bar, 50 μm. (C) Quantification of p63-positive stem cells in (A). (D) Quantification of Ki-67-positive proliferating cells in (B). Results representative of 3 analogous experiments are presented. Data represent the mean ± SD. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ against vehicle-treated mice. *Abbreviations:* DMSO = dimethyl sulfoxide; non-IR = nonirradiated.

amifostine, pose a significant clinical problem in efficiently or fully preventing mucositis (5-7). The pathogenesis of radiation-induced oral mucositis is similar in humans and mice and is composed of 4 phases: an initial inflammatory/vascular phase, an epithelial phase, a (pseudomembranous) ulcerative/bacteriological phase, and a healing phase (2). The ulceration induced by ionizing radiation on the dorsal tongue recapitulates this injury process and is frequently observed in many studies (7, 19). Herein, using a high single dose and fractioned doses of head-only irradiation in

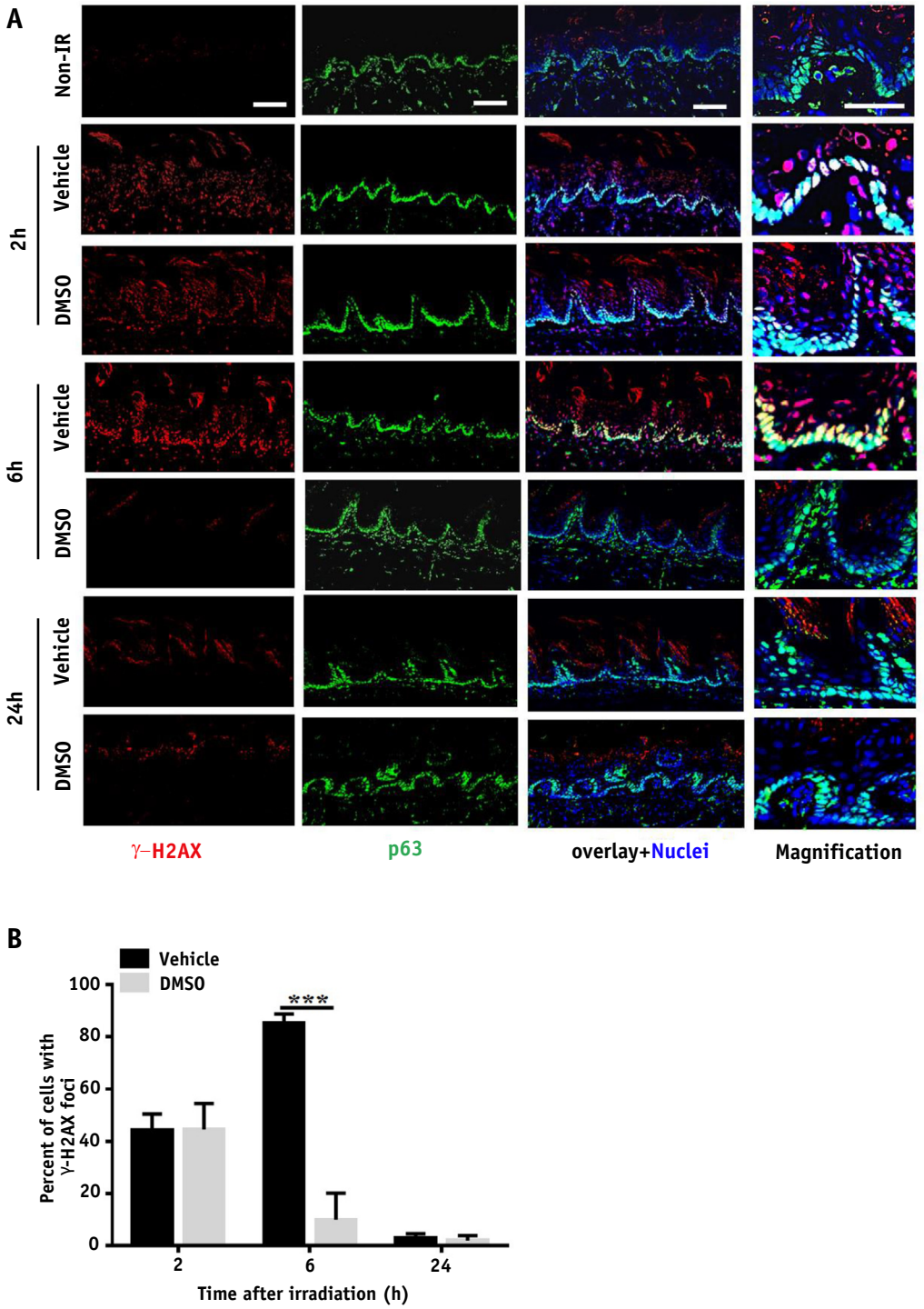

Fig. 5. DMSO repairs DNA damage of the epithelial stem cell compartment after radiation. (A) Immunofluorescence of p63 (green) and γ-H2AX (red) double-staining assay in dorsal mucosa of mouse tongue obtained at 2, 6, 24 hours (n = 4) after 16.5-Gy local irradiation to the head. Mice were treated with saline (vehicle) or DMSO (6 g/kg) 1 hour before irradiation. Tissues were counterstained with 4',6-diamidino-2-phenylindole to label nuclei (blue). The right-most side of each panels show details at higher magnification. Bar: the rightmost panel, 100 mm; the other panel, 50 mm. (B) Quantification of γH2AX-positive cells among stem cells in tongue tissue. Approximately 350 to 400 cells were scored across multiple compartments for > 2 bright foci/cell coming from at least 6 different pictures each from 4 different mice. Results representative of 2 analogous experiments are presented. Data represent the mean ± SD. ***$P < .001$ against vehicle-treated mice. *Abbreviations:* DMSO = dimethyl sulfoxide; non-IR = nonirradiated. (A color version of this figure is available at https://doi.org/10.1016/j.ijrobp.2018.07.2010.)

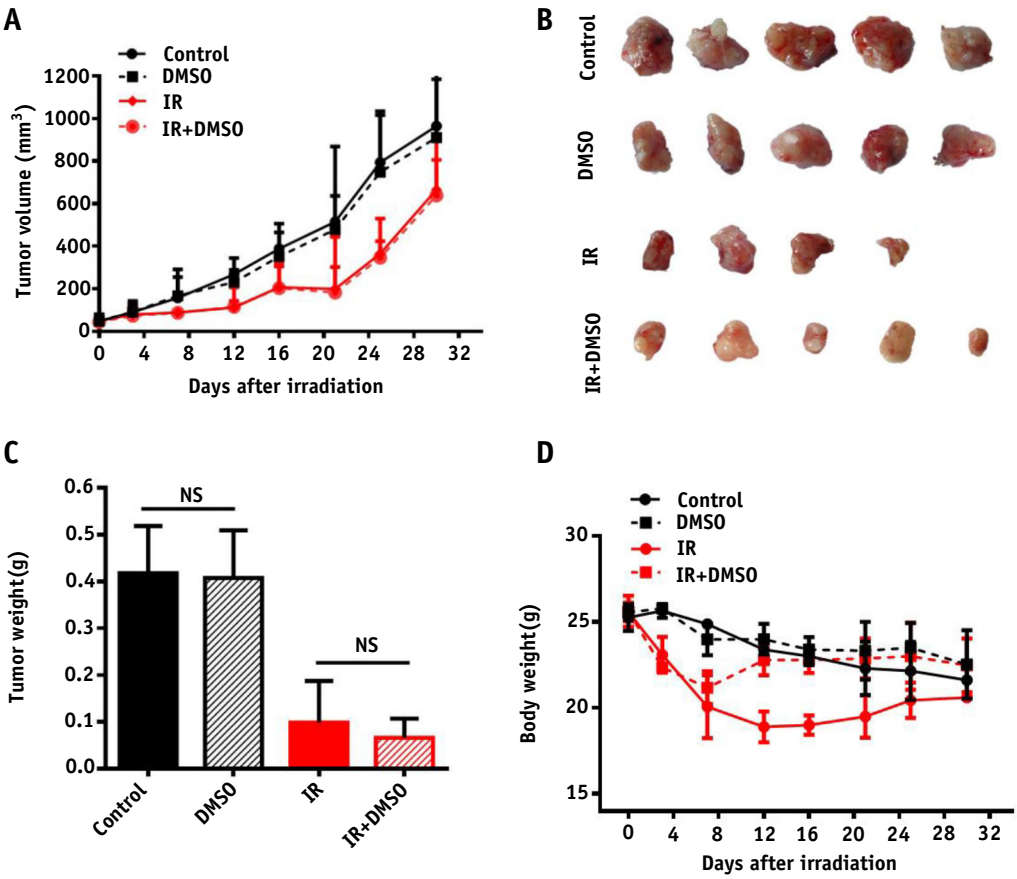

Fig. 6. DMSO has no effect on squamous cell carcinoma lines (CAL-27) tumor growth ± radiation. Human squamous cell carcinoma lines (CAL-27) cells were implanted in nude mice administered with vehicle or DMSO (6 g/kg), respectively, and received 12-Gy local irradiation to the head or no radiation. (A) Changes in tumor volumes after treatment with DMSO and radiation both individually and in combination. (B) Representative images of xenograft tumors in each group are presented on day 30. (C) Tumor weight in each group on day 30. (D) Changes in mouse body weight after treatment with DMSO and radiation both individually and in combination. Results representative of 2 analogous experiments are presented. Data represent the mean ± SD. *Abbreviations:* DMSO = dimethyl sulfoxide; non-IR = nonirradiated.

a mouse model, we show for the first time that pretreatment with DMSO protects mouse oral mucosa from severe acute radiation injury without tumor protection. More important, the efficacy of DMSO is superior to that of rhKGF and amifostine. The damage to stem cells in response to irradiation plays a key role in the mechanism inducing stem-cell depletion, thereby leading to decreased tissue regenerative capacity (19). Furthermore, our results showed that DMSO treatment in mice prevents the loss of proliferative lingual epithelial stem and progenitor cells upon irradiation, as detected by the expression of p63 and Ki-67, thereby accelerating regeneration of oral mucosal epithelium and preventing the appearance of ulcers.

Damage to cells from ionizing radiation might occur directly if energy directly ionizes or excites macromolecules in cells, or indirectly if irradiation of water-generated toxic products such as hydroxyl radicals then react with the DNA (23). DMSO has been used as a radioprotective agent because of its specific reactivity with hydroxyl radicals induced by ionizing radiation. In these studies, the concentration of DMSO (10%-15%) used was higher and very toxic (23-25). Kashino et al reported that a lower, nontoxic concentration (0.5%, 64 mM) of DMSO significantly decreased the average number of 53BP1 foci (markers of DNA DSBs) at 2 hours but not at 15 minutes after radiation in Chinese Hamster Ovary cells, compared with nontreated cells. This phenomenon was not observed in DNA repair−deficient xrs5 cells (Ku80-deficient), which indicated that DMSO at a lower concentration facilitated DNA DSB repair rather than suppressing the indirect action of irradiation (26). Recently, several studies reported that adult stem cells, such as cycling crypt base columnar cells, are relatively radioresistant, repairing DNA by homologous recombination significantly more efficiently than transit-amplifying progenitors, and DNA damage repair by homologous recombination decreases along with the differentiation of human-induced pluripotent or embryonic stem cells (27-29). By analyzing phosphorylated H2AX (another marker of DNA DSBs), we found that DMSO treatment on local irradiated mice significantly decreased

the average levels of γ-H2AX foci in p63-positive lingual epithelial stem cells at 6 hours but not at 2 hours after irradiation. Therefore, we proposed that DMSO protects against epithelial stem-cell depletion through facilitation of DNA DSB repair rather than through the suppression of indirect action of irradiation.

A major concern for the systemic delivery of DMSO is the potential risk of tumor protection from radiation therapy. Cancer cells experience high levels of defects in DNA repair. For example, more than 20% of breast cancers were reported to have a functional deficiency in the homologous recombination pathway (30, 31). As reported, DMSO exhibited significant radioprotective effects in CHO but not in xrs5 cells (which are defective in DNA DSB repair) by facilitation of DNA DSB repair (26).Consistent with these studies in vitro, we did not see any tumor-protecting ability in CAL-27 xenograft mouse models in vivo, regardless of whether it was combined with local irradiation. The molecular details of how DMSO regulates DSB repair differently between epithelial stem cells and cancer cells after irradiation needs to be investigated in the future.

Another major concern is the safety of drug administration. Unlike other drugs administered in milligrams per kilogram, DMSO is of such low toxicity that grams per kilogram are used (32). Several clinical studies reported that intravenous infusion of 20 to 60 mL DMSO per day is effective and safe for treatment of refractory pain in patients with cancer; these doses are among the effective concentrations of DMSO against radiation-induced oral mucositis based on body surface area calculation between humans and mice (33, 34).

Currently, the clinical treatment of oral mucositis is limited to supportive care aimed at managing symptoms and complications of mucositis (8). The interventions recommended or suggested for use by the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology in specific patient populations include oral care protocols, low-level laser therapy, keratinocyte growth factor-1, cryotherapy, benzydamine mouthwash, and zinc (2). No agents have been approved by the US Food and Drug Administration for the management of oral mucositis.

Conclusions

The positive results described herein suggest a potentially novel clinical application of DMSO. First, our study shows that DMSO exhibits marked protective activity against oral mucositis induced by a high single dose as well as clinically relevant fractioned doses of head-only irradiation in mice without tumor protection. Additionally, we found that the efficacy of DMSO is superior to that of rhKGF and amifostine. Furthermore, we demonstrated that DMSO treatment significantly prevents the loss of proliferative lingual epithelial stem and progenitor cells upon irradiation through facilitation of DNA DSB repair rather than through suppression of indirect action of irradiation. Finally, because of higher efficacy and poor toxicity, our findings suggest DMSO may be an optimal radioprotective agent for preventing radiation-induced oral mucositis in patients with head and neck cancers who are receiving radiation therapy.

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