The Stable Nitroxide Tempol Facilitates Salivary Gland Protection during Head and Neck Irradiation in a Mouse Model

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ABSTRACT

Purpose: Radiotherapy is commonly used to treat a majority of patients with head and neck cancers. The long-term radiation-induced reduction of saliva output significantly contributes to the posttreatment morbidity experienced by these patients. The purpose of this study was to test the ability of the stable-free radical Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), an established radioprotector, to prevent radiation-induced salivary hypofunction in mice.

Experimental Design: The heads of C3H mice were exposed to a range of single radiation doses with or without an i.p. injection of 275 mg/kg Tempol 10 min before treatment. Salivary gland output was assessed 8 weeks postirradiation.

Results: Radiation caused a dose-dependent reduction in salivary flow in this model. Tempol treatment alone significantly reduced radiation-induced salivary hypofunction. The combination of Tempol with mouth/nose shielding showed essentially complete radiation protection at 15 Gy and ~75% protection at 17.5 Gy.

Conclusions: This study demonstrates for the first time that significant radioprotection of the salivary glands is possible with Tempol in C3H mice.

INTRODUCTION

Treatment for a majority of patients with head and neck cancers includes radiation as part of their therapy (1). Salivary glands in the field of radiation are severely damaged, and radiation treatment can result in chronic salivary hypofunction (2–4). Patients experiencing reduced salivary flow suffer considerable morbidity, including dental caries, mucosal infections, dysphagia, and frank discomfort. Current management approaches are generally unsatisfactory. For example, use of conformal and intensity-modulated radiation can diminish salivary gland damage; however, it is not widely available and is expensive (5, 6). The use of sialagogues, such as pilocarpine, to increase salivary output has met with minimal success, as has the use of thiol-based radioprotectants (7, 8). Recently, however, Amifostine (WR-2721), 2-[(3-aminopropyl)amino]ethylylphosphorothioic acid, an established salivary gland radioprotectant in rats, has been approved for use in the United States for the prevention of xerostomia in head and neck cancer patients undergoing radiotherapy (9–11). Although Amifostine appears beneficial, the management of most patients with radiation-induced salivary hypofunction remains palliative in nature.

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable nitroxide that has been shown to be a radioprotector in vivo and in vitro (12, 13). It is thought that Tempol provides radioprotection by several possible mechanisms including oxidizing transition metals, mimicking superoxide dismutase activity, and scavenging free radicals (14, 15). The nitroxide form can be reduced to a nonradioprotective hydroxylamine form or oxidized to an oxoammonium cation (16), and all three forms exist in vivo (14, 16). In contrast, Amifostine is administered as an inactive prodrug requiring dephosphorylation by alkaline phosphatase for conversion into its active form (17).

A significant concern in using any radioprotective agent is the possibility that the agent will protect the tumor as well as the normal tissues (18). In animal studies, Tempol has been shown to provide radioprotection to normal tissues (12) while showing no radioprotection to tumors (19). The relatively hypoxic intra-tumor environment readily reduces the radioprotective species Tempol to its corresponding hydroxylamine form and thereby renders it useless as a radioprotector (19).

In this study, groups of C3H female mice were given a single dose of Tempol solution by i.p. injection. Ten min after Tempol administration, the mice were irradiated with single doses ranging from 5 to 20 Gy. Saliva, tissue histology, and body weight data were collected at 8 weeks postirradiation. The results demonstrate a significant radioprotective effect of Tempol on the salivary glands.

MATERIALS AND METHODS

Chemicals. Tempol was purchased from Aldrich (Milwaukee, WI) and recrystallized from diethylether. Orange-yellow needles crystallized from the supersaturated solution were filtered and dried in air. Recrystallized Tempol was stored at 4°C in sealed bottles and protected from light until use.

Animal Studies. Female C3H mice, produced by the National Cancer Institute Animal Production Area (Frederick,
MD), were used for this study. The mice were 7–9 weeks of age at the time of experimentation and weighed between 20–30 g. All experiments were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the Guide for the Care and Use Of Laboratory Animal Resource (1996), National Research Council. Salivary gland irradiation was accomplished by placing each animal into a specially built Lucite jig such that the animal could be immobilized without the use of anesthetics. Additionally, the jig was fitted with a Lucite cone that surrounded the head and prevented head movement during the radiation exposure. Lead shields designed as a part of the Lucite jigs assured that only the head of the immobilized animal was irradiated. For some studies, an additional lead shield was included to protect the anterior aspect of the mouth. Single radiation doses ranging from 5 to 20 Gy were delivered to the head of the animal by a Therapax DXT300 X-ray irradiator (Pantak, Inc., East Haven, CT) using 2.0 mm Al filtration (300 Kilovoltage Peak) at a dose rate of 1.9 Gy/min. For animals receiving Tempol treatment, a dose of 275 mg/kg (in 100 μl of sterile water) was injected i.p. 10 min before radiation treatment. Immediately after irradiation, animals were removed from the Lucite jig and housed (5 animals/cage) in a climate and a light/dark-controlled environment and allowed free access to food and water.

Sample Collection. Saliva samples were collected 8 weeks postirradiation. The 8 week time point for data collection was chosen based on studies in rats by Nagler et al. (20, 21). Nagler et al. (21) demonstrated that observations ≥14 days postirradiation were inadequate to assess true radiation-induced hyposalivation in rats. In contrast, they also showed separately that at 3 months after irradiation with 15 Gy, salivary function in both the parotid and submandibular glands of rats was dramatically impaired (20, 22, 23). The 8 week time point, although an estimate, seemed likely to provide reliable data at a convenient interval. Mice were weighed and given ketamine (100 mg/ml) and Rompun [xylazine (20 mg/ml)] in sterile water by i.p. injection (10 μl/10 g; Phoenix, St. Joseph, MO). A fresh solution of pilocarpine (0.5 mg/ml; Sigma, St. Louis, MO) was made using PBS. A total of 25 μl was administered s.c. per animal. Saliva collection began within 2 min of pilocarpine administration. Animals were positioned with a 75-mm hematocrit tube (Drummond, Broomall, PA) placed in the oral cavity, and whole saliva was collected for 10 min into preweighed 0.75-ml Eppendorf tubes (24). The amount of saliva collected was determined gravimetrically. Immediately after saliva collection, anesthetized animals were euthanized by cervical dislocation, and the bilateral submandibular glands were removed. The glands were immediately placed in 4% paraformaldehyde at room temperature, processed, and blocked in paraffin, and 5-μm sections were obtained and stained with H&E for light microscopic examination.

Statistical Analysis. Descriptive statistics were calculated, including the means and SDs. Both the Student t test and a generalized linear model were applied to the data, where the salivary output was the response variable. Putative explanatory variables, including radiation dose, treatment with Tempol, and the use of shielding, and their interaction terms were entered into the generalized linear model.

RESULTS

Tempol Treatment in the Absence of Radiation Does Not Affect Salivary Output. Initially, we wanted to determine whether Tempol in the absence of radiation could affect saliva output. To accomplish this, we compared saliva production in female C3H mice 8 weeks after administration of Tempol with that of untreated control mice (Fig. 1). The Tempol-treated mice, on average, show a reduction in saliva output, but it was not statistically significant (t = 1.80; P = 0.11), 190 ± 9.28 versus 140.20 ± 9.87 μl. We conclude that there was no statistically significant difference in saliva production detected in mice treated with Tempol versus the untreated controls.

Tempol Administration Provides Radioprotection for Salivary Glands. To determine whether Tempol affords any radioprotection for salivary glands, C3H mice, with or without Tempol treatment, were irradiated with 0, 5, 12.5, 15, 17.5, or 20 Gy. Over this radiation dosage range, no significant effect on animal weight was observed. Saliva was collected 8 weeks postirradiation, as described above. Saliva production in C3H mice was sensitive to radiation in a dose-dependent manner (Fig. 2). A dose of 5 Gy was without any effect on salivary flow, but a significant reduction in saliva output occurred from 12.5 through 20 Gy (all t > 3.64; P < 0.007; Fig. 2). Mice treated with Tempol exhibited significantly less reduction in saliva flow compared with untreated irradiated mice (P = 0.01). As shown in Fig. 3, Tempol-treated mice irradiated with doses of 12.5 and 15 Gy, without additional mouth and nose shielding, showed ~20–30% decrease in salivary flow, which was substantially improved when compared with mice irradiated without Tempol (~50–60% decrease). Radiation-induced reduction in saliva flow increased to ~50% and 75% in Tempol-treated animals exposed to 17.5 and 20 Gy, respectively. The interaction of Tempol and radiation is significant (P = 0.0004). We conclude that the use of Tempol alone provides significant radioprotection in mice.

Tempol in Combination with Additional Mouth/Nose Shielding Provides Increased Radioprotection for Salivary Glands. Next, we wanted to determine whether administration of Tempol, in combination with additional mouth/nose lead shielding, would afford further radioprotection for salivary glands. To address this, we irradiated C3H mice with 0, 15,
17.5, or 20 Gy as described (see “Materials and Methods”). The combination of Tempol plus mouth/nose shielding (Fig. 4) dramatically enhanced salivary flow in the irradiated animals ($P < 0.0001$). No statistically significant reduction in saliva production was observed in mice irradiated at 15 or 17.5 Gy (all $t < 1.2; P > 0.1$; Fig. 4). Interestingly, animals irradiated without Tempol showed no statistically significant benefit to the use of mouth/nose shielding.

**Histology.** To determine the morphological status of glands from control and irradiated mice, we analyzed two sections from each sample under light microscopy with H&E staining. Two months after irradiation, no significant loss of cells or inflammatory infiltrate was observed. In addition, we did not note any features of necrosis or apoptosis in these samples. Lastly, no degenerative changes or fibrosis was observed in the irradiated tissues. At this time point, there were no apparent histological changes in the salivary glands between the irradiated groups and the control mice, even at 20 Gy of irradiation (data not shown).

**DISCUSSION**

Earlier observations that Tempol could afford radiation protection *in vivo* were not unexpected because nitroxides had been shown to be effective *in vitro* against several types of reactive oxygen species (13, 25, 26). Because of these earlier results, we decided to test the hypothesis that Tempol could protect salivary glands from functional damage due to head and neck irradiation. Our findings indicate a dramatic effect of Tempol on reducing radiation-induced salivary hypofunction, despite a small (but statistically insignificant) hyposalivatory effect of the agent alone in the absence of radiation. The dose of Tempol used was $\approx 80\%$ of the LD$_{50}$ (12). Nonetheless, there were no adverse effects resulting from Tempol treatment, as evidenced by that fact that (a) all of the mice survived the Tempol treatment, and (b) no significant difference in body weight was seen for animals given Tempol versus control animals (data not shown).

We found a radiation dose-dependent inhibition of saliva flow in the C3H mouse model (Fig. 2). Although the radiation dose response for saliva output in rats has been well studied, relatively little study exists with mouse models (20, 22, 23, 27). Recently, two studies using murine models have measured saliva output up to 4 weeks postirradiation (28, 29). Comparisons of the Lin et al. (28) report and our study are limited because the mice in that study were exposed to only a single full-body dose of 30 Gy. In addition, Lin et al. (28) collected pilocarpine-stimulated saliva up to 72 h immediately after irradiation without anesthesia, whereas we collected pilocarpine-stimulated saliva after 8 weeks in anesthetized animals. Takeda et al. (29) demonstrated partial recovery of the salivary flow in postirradiated mice by blocking synthesis of nitric oxide (29). They observed a modest but significant increase in salivary output in mice given an inhibitor of nitric oxide synthesis at 1 week postirradiation at 15 Gy. An important distinction between that study and our report is that Takeda et al. (29) used a treatment (postirradiation) model, not a prevention model. They administered the nitric oxide synthesis inhibitor to mice that had been previously (1 week) irradiated with 15 Gy to study inflammation-mediated nitric oxide-induced damage. In contrast, we used Tempol as a radioprotectant with salivary output measured at 8

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**Fig. 2** The effect of radiation dose on salivary flow rate. Animals were irradiated without Tempol treatment. Each value represents the average from five animals ± SE. The overall difference was significant when analyzed by generalized linear model ($P < 0.0001$).

**Fig. 3** The effect of Tempol on radiation-induced reductions of salivary flow rate. Animals were untreated (■) or given a single i.p. dose of Tempol (275 mg/kg) 8 weeks previously (□). Each value represents the average from five animals ± SE. The overall difference was significant when analyzed by generalized linear model ($P = 0.0004$).

**Fig. 4** The effect of Tempol plus radioprotective mouth/nose shielding on radiation-induced reductions of salivary flow rate. Animals were untreated (■) or given a single i.p. dose of Tempol (275 mg/kg) 8 weeks previously (□). Each value represents the average from five animals ± SE. The overall relationship was significant when analyzed by generalized linear model ($P < 0.0001$).
weeks postirradiation. In studies of radiation effects on rat salivary gland function, acute measurements of salivary output do not necessarily indicate long-term sequellae (20–23). Our results suggest that the observed radiation-induced loss of salivary function is likely because of subtle (subcellular and molecular) changes in the glands because at the 8 week time point, no microscopic changes in the gland tissues were noted. This lack of significant morphological alteration is in agreement with many earlier reports in rats (23, 30–32). However, unlike the recent study in rats by Takagi et al. (33), we did not observe any significant loss of cells in irradiated samples, vacuolation in the acinar cells, or enlargement of the acinar lumen. It should be noted, however, that our histological observations were done at a considerably later time point than the Takagi et al. (33) study (2 months instead of 2 weeks). In addition, Paardekooper et al. (30) demonstrated that radiation-induced apoptosis occurs in rat salivary glands within hours after exposure to a radiation dose of ≥2 Gy. Interestingly, at 6 days after exposure to a radiation dose of 15 Gy, no significant cell loss in the rat parotid gland was observed, although the function of the gland as measured by flow rate was reduced by 50% (30, 34).

Tempol was clearly able to act as an effective radioprotector for salivary glands (Figs. 3 and 4). Tempol afforded protection in mice irradiated both with and without the additional mouth/nose shielding. However, we found a remarkable degree of radioprotection when the combination of Tempol plus the mouth/nose shield was used. For example, at a dose of 15 Gy, the Tempol-treated mice exhibited an average salivary output identical to that of the nonirradiated group given Tempol (Fig. 4). Even at a single dose of 17.5 Gy, saliva production was only 30%, 34).

Comparisons of the relative effectiveness of Tempol with Amifostine as a salivary gland radioprotectant are difficult because rat models were used in previous studies (9, 10, 35). For the present purpose, we determined the relative protective factor of Tempol, the ratio of the percentage reduction for nonprotected/protected animals, using total saliva output (data not shown) as the parameter examined (10). Menard et al. (10) exposed rats with and without Amifostine to 15.3 Gy of whole-head irradiation and collected parotid saliva at 6–10 days post-treatment. The relative protective factor for Amifostine using total volume parotid saliva collected was 2.1 (10). Using whole saliva collected 8 weeks posttreatment, we found that the relative protective factor for Tempol was 2.2 in the C3H mouse given a radiation dose of 15 Gy. Whereas the numbers for Amifostine and Tempol seem to compare favorably, any real comparison of these values is impossible because of differences in experimental model and design.

Recent evidence indicates that maintaining the integrity of the endothelial lining of the local capillaries may be particularly important to maintaining organ viability after irradiation (36). Because Tempol was administered systemically (i.p.), it is possible that the observed radioprotection by Tempol may be a result of a combination of effects on capillary endothelial cells in addition to salivary epithelial cells. Tempol given i.p. may have induced systemic mediators or actions that could have led to radioprotective effects on the salivary glands. Salivary acinar cells are considered more radiosensitive than the ductal cells, and indeed acinar cells are the only salivary parenchymal cell type physiologically capable of secreting fluid (37, 38). We suggest that future studies examining both parenchymal cell and endothelial cell integrity immediately after irradiation (within the first 24 h) may be helpful in understanding the damage mechanisms involved.

There are several mechanisms by which Tempol could protect salivary gland function in the present study. Damage by ionizing radiation, for the most part, is caused by ionization of water to produce free radicals such as ‘H, ‘OH, and aquated electrons. These highly reactive radicals react rapidly in a non-discriminate manner with other cellular constituents, causing damage and generating secondary free radicals, including superoxide, with varying life times (39). Scavenging of the primary radiolysis products such as hydroxyl radicals by scavengers such as aminothiols and other reducing agents may prevent damage because the chemical reaction proceeds at diffusion-limited rates; however, in practice, this is difficult to realize in terms of achieving sufficient scavenger concentrations at critical target sites. Nitroxides, which act as superoxide dismutase mimics, can specifically react with superoxide and effectively convert superoxide to hydrogen peroxide (16, 40). It is thought that the hydroxyl radical is the primary damaging species produced by ionizing radiation. However, several studies have shown that cells overexpressing superoxide dismutase activity are less responsive to cell killing from radiation, which suggests a role for superoxide in cellular damage (41, 42). Several types of secondary free radicals including carbon-centered radicals and organoperoxides are generated by reactions with primary radiolysis products (39). Whereas these species may be less reactive than hydroxyl radical, they can nonetheless induce damage. In general, reducing agents such as thiols (including the metabolic product of Amifostine) may scavenge these secondary radicals; nitroxides, being radicals themselves, may be more efficient in scavenging these longer-lived radicals by participating in radical-radical recombination and/or electron exchange reactions (14, 15). Moreover, Tempol has been shown to interrupt Fenton type chemistry and hence protect against secondary and tertiary reactive species produced by ionizing radiation (13–15). Given that aminothiols and nitroxides may have overlapping and slightly different mechanisms of action with respect to radioprotection, it is possible that combination of the two might afford additive protection. We are currently testing this hypothesis.

A major concern of systemic delivery of radioprotective agents is tumor protection. There was considerable debate during the 1980s as to whether Amifostine protects tumor from radiation damage (43–46). Because Amifostine has moved into numerous clinical trials and was recently approved for use in head and neck radiation treatments, this debate continues (11, 47). Nonetheless, Amifostine has demonstrated the ability to protect against radiation-induced xerostomia in clinical trials (11). The potential advantages of considering another protector such as Tempol are two-fold. First, Tempol has been shown in the present study to protect salivary gland function, and other studies have shown protection for bone marrow (12) and radiation-induced alopecia (48). Second, Tempol may possibly provide selective normal tissue radioprotection. Because the pres-
ence of hypoxia (reducing microenvironment) characterizes most solid tumors, Tempol would be expected to be reduced at a faster rate than for normal tissues (19). The reduction product of Tempol (hydroxylamine) is not a radioprotector (13). Hence, after injection of Tempol into a tumor-bearing animal, there should be a time when the amount of Tempol in normal tissues exceeds that in tumor. Tempol has already been shown not to protect tumor cure for single-dose radiation treatment using the same concentration of Tempol and timing as used in the present study (19). Preliminary tumor radiation regrowth delay studies have indicated that Tempol does not protect when given 10 min before five daily radiation fractions.3 More work is required to further substantiate the hypothesis that Tempol will selectively protect normal tissues against radiation damage.

In conclusion, we have shown that a single dose of Tempol administered i.p. immediately before irradiation, in conjunction with mouth/nose shielding, is dramatically protective against radiation-induced xerostomia following radiation therapy for oral cavity cancer. Tempol may offer possible salivary protective benefits for individuals undergoing head and neck irradiation.

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