

Kinetics of Tempol for Prevention of Xerostomia Following Head and Neck Irradiation in a Mouse Model

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Abstract Purpose: Radiotherapy is commonly used to treat the majority of patients with head and neck cancers. Salivary glands in the radiation field are dramatically affected by this procedure. The purpose of this study was to examine pharmacokinetic characteristics of the stable nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (tempol) with respect to radioprotection of the salivary glands.

Experimental Design: To evaluate the effect of different doses and times of administration, the heads of C3H mice were exposed to a single irradiation dose of 15 Gy, with i.p. tempol injection. To analyze other routes of administration, we injected 275 mg/kg tempol by an i.m., i.v., or s.c. route, 10 minutes before irradiation. We also tested whether oral administration of tempol in a topical form (either in a mouthwash or gel) provided any salivary gland protection.

Results: Tempol treatment (137.5 or 275 mg/kg, i.p., 10 minutes before irradiation) significantly reduced irradiation-induced salivary hypofunction (~50-60%). I.v. or s.c. administration of tempol also showed significant radioprotection, whereas i.m. administration proved to be ineffective. Topical use of tempol, either as a mouthwash or gel, also was radioprotective.

Conclusions: Our results strongly suggest that tempol is a promising candidate for clinical application to protect salivary glands in patients undergoing radiotherapy for head and neck cancers.

Oral and laryngeal cancer will affect 41,000 Americans this year and >350,000 new cases will be diagnosed worldwide (1, 2). The curative treatment modalities consist of surgery and/or irradiation. Chemotherapy can be added to this regimen to decrease the possibility of development of distant metastasis (1, 3). Salivary glands in the irradiation field are severely damaged and, consequently, this results in marked salivary hypofunction in ~80% of patients (4-7). Patients experiencing reduced salivary flow suffer considerable morbidity, including dental caries, mucosal infections, dysphagia, and extensive discomfort. Current management approaches remain palliative and are generally unsatisfactory. Although Amifostine (WR-2721; 2-[(3-aminopropyl)amino] ethylphosphorothioic acid), a radioprotector, has been recently approved for use in prevention of xerostomia in head and neck cancer patients

undergoing radiotherapy (8, 9), controversy remains as to whether Amifostine also confers tumor protection (10, 11).

Recently, we have shown (12) that tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a stable nitroxide, was able to successfully protect murine salivary glands from irradiation. In this first study, we administered tempol to C3H mice 10 minutes before irradiation using an i.p. injection, an undesirable method of administration in a clinical setting. In the present study, using this same animal model, we have further examined the radioprotective properties of tempol for salivary glands, examining doses, times, and other routes of administration for potential clinical application.

Materials and Methods

Chemicals. Tempol (Aldrich, Milwaukee, WI) was recrystallized from diethylether; orange-yellow needles obtained from the supersaturated solution were filtered and air-dried. Recrystallized tempol was stored at 4°C in sealed bottles and protected from light until use. The gel form of tempol was made by Starks Associates, Inc. (Buffalo, NY), by adding tempol to a metasilose base gel at a final concentration of 470 mmol/L. The mouthwash consisted of 470 mmol/L tempol dissolved in a solution containing 0.12% of chlorhexidine gluconate (Alpharma USPD, Inc., Baltimore, MD).

Animal irradiation. Female C3H mice, bred in the National Cancer Institute Animal Production Area (Frederick, MD), were used for this study. The mice were 7 to 9 weeks of age at the time of experimentation and weighed between 20 to 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 Animals (1996), National Research

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Council. Irradiation of salivary glands was accomplished by placing each animal into a specially built Lucite jig in such way 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 irradiation exposure. Single irradiation doses at 15 Gy were delivered to only the animal's head by a Therapax DXT300 X-ray irradiator (Pantak, Inc., East Haven, CT) using 2.0-mm Al filtration (300 kVp) at a dose rate of 1.9 Gy/min. This irradiation dose leads to significant (60%) loss of salivary flow (12). Immediately after irradiation, animals were removed from the Lucite jig and housed (five animals per cage) in a climate and light-controlled environment and allowed free access to food and water.

Time of administration, dose dependence, and systemic administration experiments. For each time, dose, or route of administration, a group of four animals was used and all *in vivo* experiments were done twice. To assess the effect of different times of injection, 275 mg/kg (in 100 μ L sterile water) tempol was injected i.p. either 5 or 10 minutes before, or 10 minutes after, 15 Gy. To assess the radioprotective effect of different doses of tempol, we used the following doses: 0, 27.5, 70, 137.5, and 275 mg/kg body weight. All doses were injected i.p., 10 minutes before 15 Gy. To study radioprotective effects of tempol when administered systemically by different routes, a single dose (275 mg/kg) of tempol was injected either i.p., i.v. (tail vein), i.m. (hind limb muscle), or s.c., 10 minutes before 15 Gy.

Topical oral administration. Tempol gel, or placebo gel containing only vehicle, was administered topically to the oral cavity of mice before irradiation. Briefly, mice were anesthetized and 50 μ L tempol gel was applied to a sterile cotton ball, small enough to fit into a mouse's oral cavity. This impregnated cotton was left in place for either 10 or 20 minutes. Additionally, tempol dissolved in a chlorhexidine mouthwash was tested. Fifty microliters of a 470 mmol/L solution was applied to a sterile cotton ball and administered following the same approach as for the gel application.

Saliva collection. To determine salivary flow rate, saliva samples were collected 8 weeks after irradiation (12). Mice were weighed and mild anesthesia was induced with a solution of ketamine (100 mg/mL, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (20 mg/mL, Phoenix, St. Joseph, MO) in sterile water, given i.p. (1 μ L/g of body weight). Whole saliva was collected after stimulation of secretion, using pilocarpine (0.5 mg/kg body weight) administered s.c. Saliva collection began within 2 minutes 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 in preweighed 0.75-mL Eppendorf tubes for 10 minutes. The amount of saliva collected was determined gravimetrically. Immediately afterwards, anesthetized animals were euthanized by cervical dislocation.

Statistical analysis. Descriptive summaries of our findings (means \pm SE) were derived and reported. A general linear model (ANOVA) was used to analyze the salivary response variables. Putative explanatory variables included radiation, type of treatment (time and dose of tempol) administered, together with their first-order interaction terms, were included in the models. PROC GLM in SAS, version 9.1 was used to do the analyses. Within each ANOVA model, group comparisons were made using the LSMEANS procedure. Due to the multiplicity of comparisons that were made, we used $\alpha = 0.01$ per comparison as a compromise to offset the potential inflation in type I error for the study. That is, we consider effects having $P < 0.01$ as significant in this analysis.

Results

Eight weeks after irradiation (15 Gy), salivary flow was determined in mice receiving either tempol or no treatment before irradiation. Control, unirradiated animals had a mean whole saliva output of 289 ± 28 μ L in 10 minutes, whereas mice treated with 15 Gy showed a mean salivary flow of 126 ± 22 μ L in 10 minutes. This represents an average reduction in

salivary flow of 58%. In our previous study (12), we found a similar reduction in salivary output after 15 Gy.

Effect of tempol dose on salivary gland radioprotection. We evaluated the ability of several different doses of tempol to prevent irradiation-induced reduction in salivary flow. In these experiments, the irradiation-only group showed a salivary reduction of 58% (Fig. 1). No statistically significant protection was seen when mice received doses of 27.5 or 70 mg/kg of tempol i.p., which led to reductions in salivary output of 68% and 57%, respectively. However, when mice were treated with 137.5 mg/kg tempol, half the dose previously shown to protect salivary glands (12), salivary flow was reduced 34%, significantly different when compared with untreated, irradiated mice ($P < 0.003$). In this same experiment, animals receiving the 275 mg/kg dose of tempol i.p. also exhibited significant protection of salivary flow (31% reduction), when compared with the untreated irradiated group ($P < 0.001$; Fig. 1).

Effect of tempol administration time. To evaluate the effect of different administration times on tempol's efficacy, mice were treated with i.p. injections of 275 mg/kg tempol either 10 or 5 minutes before, or 10 minutes after, 15 Gy and salivary flow rate was examined after 8 weeks. Compared with untreated irradiated mice, i.p. tempol injections alone, either 5 minutes before irradiation or 10 minutes after irradiation, did not result in any significant protection of salivary flow (Fig. 2; reduction of $44 \pm 5.2\%$, $P < 0.2$ and $48 \pm 8.4\%$, $P < 0.4$, respectively). However, when tempol was administered 10 minutes before irradiation, the mice showed a mean reduction in salivary flow of $22 \pm 6.2\%$, $P < 0.01$ (Fig. 2).

Effect of other routes of tempol administration. To evaluate other routes of tempol administration, expected to be more appropriate in a clinical setting, we examined the administration of tempol (275 mg/kg, administered 10 minutes before irradiation) either via i.p., i.v., i.m., or s.c. injection. In this experiment, the average salivary flow rate in the control group (i.e., nontreated, non-irradiation mice) was 277 ± 18 μ L, whereas for the group that received irradiation, but no tempol treatment, it was 120 ± 20 μ L, a reduction of $\sim 60\%$ ($P < 0.004$; Fig. 3). Intramuscular tempol administration led to an average saliva production of 159 ± 12.4 μ L (42% reduction from control), which was not different from the untreated irradiation group ($P < 0.07$). In contrast, when tempol was administered either i.v. or s.c., mice showed a significantly

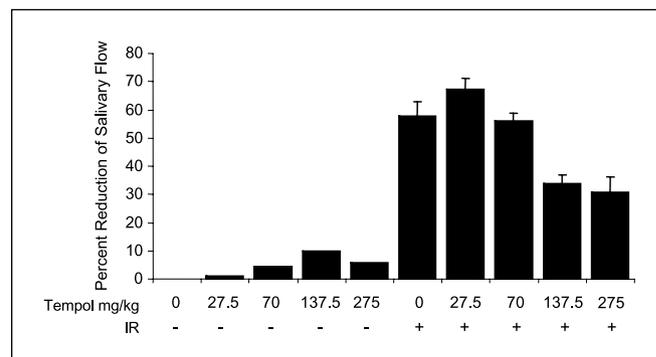


Fig. 1. Effect of different tempol doses on salivary gland radioprotection. Mice received different doses of tempol via i.p. injection with or without irradiation (IR) as indicated. Columns, mean percent reduction in salivary flow in 10 minutes ($n = 8$); bars, \pm SE. Only 137.5 and 275 mg/kg conferred radioprotection ($P < 0.003$ and $P < 0.001$, respectively).

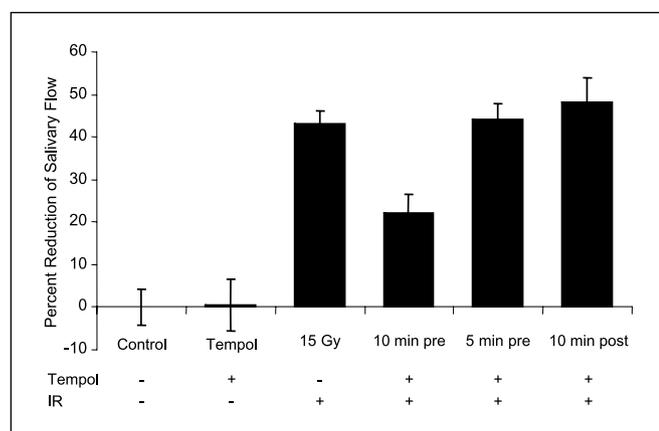


Fig. 2. Effect of time of tempol administration on salivary radioprotection. Tempol (275 mg/kg) was administered by i.p. injection at the times indicated. Radioprotection was only seen when administered 10 minutes before irradiation (IR). Columns, means ($n = 8$); bars, \pm SE.

higher salivary output. As shown in Fig. 3, for mice receiving the i.v. tempol administration the mean salivary flow rate was $183 \pm 17 \mu\text{L}$, $\sim 66\%$ of control values ($P < 0.005$), whereas for mice receiving the s.c. administration, it was $223 \pm 12 \mu\text{L}$, $\sim 80\%$ of control values ($P < 0.0001$). The s.c. route was statistically as effective as i.p. tempol administration, which showed an average salivary output in these experiments of $233 \pm 16 \mu\text{L}$, a reduction of 16% compared with untreated irradiated mice.

Topical application of tempol. Finally, we determined if topical application of tempol in the oral cavity would provide any radioprotection of salivary glands. In principle, this form of administration would be easy to employ in the clinic and oral mucosa exhibits high levels of drug permeability (13, 14). To address this issue, we used a metasilose base gel in which 470 mmol/L tempol was dissolved, as well as a formulation of 470 mmol/L tempol dissolved in chlorhexidine, a commonly used antiseptic mouthwash. The control groups included mice receiving the tempol gel but no irradiation exposure, mice receiving the placebo gel with and without irradiation exposure, mice receiving tempol in chlorhexidine without irradiation exposure, and mice receiving chlorhexidine alone with and without irradiation exposure. When applied 10 minutes before irradiation, neither topical formulation provided significant radioprotection ($P < 0.2$ for both; data not shown). However, when the time of application was extended to 20 minutes before irradiation, both afforded a significant protective effect on salivary output measured 8 weeks after irradiation (Fig. 4). For example, in these experiments, compared with the untreated nonirradiated mice, mice just exposed to 15 Gy showed a 60% decrease in salivary flow. However, the irradiated group that received tempol diluted in chlorhexidine, had an average salivary flow rate of $196 \pm 11 \mu\text{L}$, a reduction of only 29%, ($P < 0.0008$), whereas irradiated mice receiving the tempol gel exhibited a salivary flow of $219 \pm 12 \mu\text{L}$, a reduction of only 21% ($P < 0.0001$; Fig. 4).

Discussion

We conducted the present study based on our earlier findings that the stable nitroxide tempol when administered i.p. led to

radioprotection of murine salivary glands. We have examined the effects of tempol dose, the route of tempol administration, and the timing of tempol administration in this model. In the previous study, the dose used (275 mg/kg tempol) was 75% of the LD_{50} when administered i.p. Thus, we initially preformed studies to examine the tempol dose dependence of salivary gland radioprotection. We found equivalent irradiation protection of salivary output after tempol doses of 275 and 137.5 mg/kg body weight. There was no significant protection at lower doses.

Next, we evaluated the significance of the time of tempol i.p. administration. Of the three timepoints tested, only administration 10 minutes before irradiation was effective in protecting salivary gland function. Neither administration 5 minutes before irradiation nor 10 minutes after irradiation was useful. We speculate that this result is a reflection of optimal tissue and blood levels of tempol, as well as pharmacokinetic variables appropriate for tempol's efficient interaction with irradiation-induced reactive oxygen species. Tempol reacts with these reactive oxygen species, leading to its reduction. It is thought that tempol provides radioprotection by several possible mechanisms, including oxidizing transition metals, mimicking superoxide dismutase activity and scavenging free radicals (15, 16). Both serum and tissue drug levels are commonly used to assess *in vivo* pharmacokinetics. However, in mice, there is a small total circulating blood volume and limited invasive access, both of which make it difficult to conduct repeat sampling and/or real-time measurement of circulating blood levels of a drug. Tempol, as with several other nitroxyl radicals, is rapidly reduced in living tissues. Electron paramagnetic resonance spectrometer measurements of active tempol have shown sufficient blood activity levels, when administered i.v., maximally 10 minutes before irradiation (17, 18). Therefore, we chose not to test times for systemic tempol administration beyond 10 minutes.

For clinical applications, an i.p. route of administration is undesirable. Accordingly, we examined salivary gland protection following tempol administration by other routes: i.m., i.v., s.c., and intraoral topical application. We speculate that the i.m. route did not provide efficient absorption of tempol given the single time point assayed. On average, we did see higher salivary flow rates with i.m. tempol injection; however, the results were not statistically significant ($P < 0.07$). I.v. administration led to a significantly increased salivary flow rate ($P < 0.005$) but less on average than occurred following s.c.

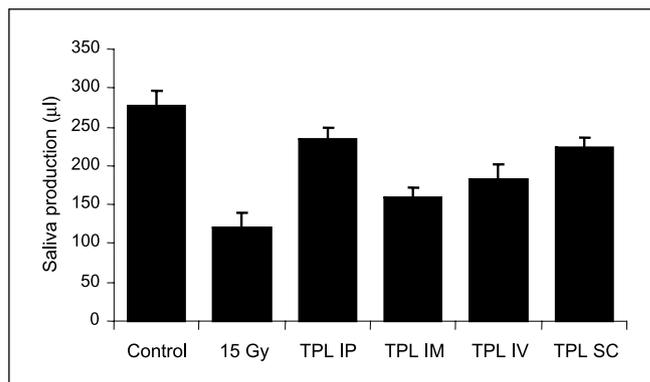
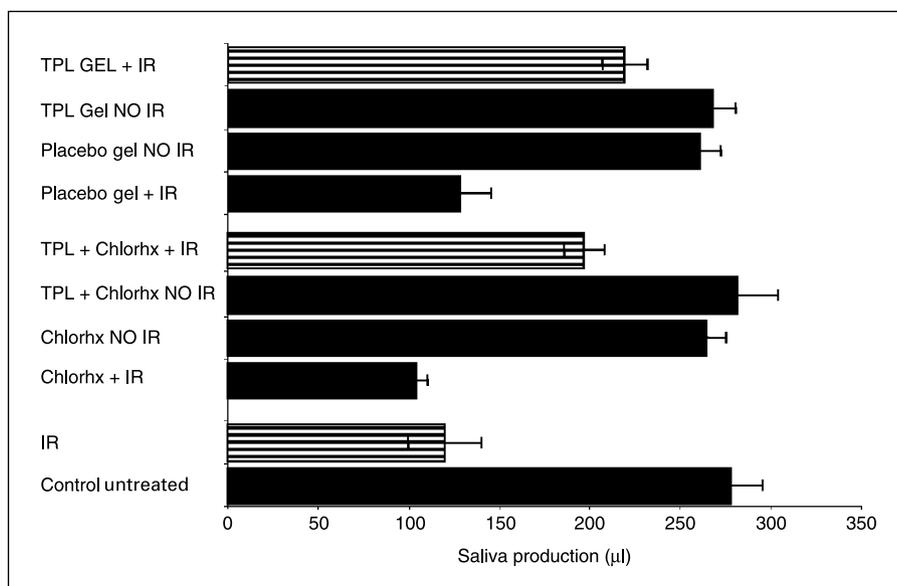


Fig. 3. Effect of different routes of tempol administration on salivary production in irradiated mice. Mice were administered tempol (TPL; 275 mg/kg) by either i.p., i.m., i.v., or s.c. injection. Columns, means for saliva production over 10 minutes ($n = 8$); bars, \pm SE.

Fig. 4. Effect of topical intraoral administration of tempol on salivary radioprotection. Tempol (TPL) was used in a gel form or diluted in chlorhexidine (Chlorhx) and applied intraorally 20 minutes before irradiation (IR). Mice that received tempol diluted in chlorhexidine before irradiation had an average salivary flow of $196 \pm 11.3 \mu\text{L}$, a reduction of only 29% in the salivary flow rate ($P < 0.0008$), whereas mice receiving the tempol gel before irradiation exhibited a salivary flow of $219 \pm 12.6 \mu\text{L}$, a reduction of only 21% in the salivary flow rate ($P < 0.0001$). Columns, means for saliva production over 10 minutes ($n = 8$); bars, \pm SE.



tempol administration. It is likely that for the time point studied herein, s.c. delivery was the most effective systemic route, providing equivalent radioprotective results to i.p. administration. It is interesting that topical intraoral tempol application was also radioprotective. We assume that tempol, when applied topically, was readily absorbed in the bloodstream and accumulated in the salivary gland in a time-dependent way. Such topical administration is noninvasive and thus appealing. However, due to the extent of time needed for effective topical application (i.e., 20 minutes), we conclude that s.c. delivery would be a preferable route for clinical use. The s.c. route offers more drug delivery control, and when the therapeutic window is small, as is the case with tempol, this may be the most important factor to consider.

A major concern with systemic delivery of radioprotective agents is possible tumor protection. For example, Amifostine has the ability to protect against radiation-induced xerostomia in clinical trials (9), but the debate continues on whether Amifostine confers tumor radioprotection (10). Thus, it is reasonable to consider another radioprotector such as tempol for radiation-induced xerostomia. Tempol has been shown to protect salivary gland function (12) and bone marrow (19) and to prevent radiation-induced alopecia (20, 21). Tempol has also been shown to increase tumor free survival in *Atm*-deficient mice (22) and in C3H mice (23). Furthermore, it was recently shown that the treatment of *Atm*-deficient mice with tempol increases latency to tumorigenesis and that this

chemopreventative effect is associated with reduced oxidative damage and stress (23). Additionally, Erker et al. recently showed that tempol has significant chemopreventative effects on two cancer-prone mouse models, *Atm*^{-/-} and *p53*^{-/-} mice, and also provided evidence that tempol modulates redox-mediated cellular signaling partly through activation of the p53 pathway (24). Importantly, tempol may provide selective normal tissue radioprotection. Because most solid tumors are hypoxic (i.e., a reducing microenvironment), tempol would be expected to be reduced at a faster rate in tumors than in normal tissues (25), and the reduction product of tempol (hydroxylamine) is not a radioprotector (26).

In summary, using a murine model of irradiation-induced salivary hypofunction, we have shown that administration of tempol leads to salivary gland radioprotection in a manner that may be clinically useful. Tempol's effects are both dose and administration time dependent, and both s.c. and topical intraoral administration routes are quite effective. Although we have speculated that our results may offer potential applications in the clinic for radiotherapy patients, it is important to remember that for these animal experiments, a single, relatively high, irradiation dose was used. In contrast, patients commonly receive a series of fractionated irradiation doses, which more likely will require a different tempol dosage treatment regimen with multiple administrations. Nonetheless, the present results suggest the value of exploring clinical radioprotection studies with tempol.

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