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ORIGINAL RESEARCH

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Nigella sativa oil mitigates xerostomia and preserves salivary function in radiotherapy-treated mice

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Abstract

Objective: This study aimed to assess if *Nigella sativa* oil (NSO), a health supplement containing thymoquinone as a major component, can act as a protective agent in salivary gland stem cells following radiotherapy (RT) damage.

Methods: Forty, 10-week-old, male C3H/HeJ mice were randomized to four experimental groups: sham RT + H_2O gavage (control) ($N = 4$); 15 Gy RT + H_2O gavage ($N = 12$); sham RT + NSO gavage ($N = 12$); and 15 Gy RT + NSO gavage ($N = 12$). Weight changes, saliva production, and salivary gland histopathologic staining were recorded for each group over the course of the experiment.

Results: All mice in the sham RT + H_2O gavage and sham RT + NSO gavage groups demonstrated 100% 60-day survival. RT + H_2O compared to RT + NSO gavaged mice were significantly underweight by an average of 6.4 g ($p < .001$). Salivary output showed significant decline in RT + H_2O gavaged mice at days 3 and 16, whereas salivary output in RT + NSO during these same time periods was comparable to the control. At day 60, all mice that survived recovered salivary function regardless of their treatment arm. Salivary specimens from the RT + NSO gavage group demonstrated early signs of recovery of Kr 5+ salivary gland stem cells in both submandibular and sublingual glands at day 16 with complete recovery by day 60, marked by strong histopathologic staining, whereas the RT + H_2O gavage group did not recover as effectively.

Conclusion: NSO may help preserve salivary function in mice treated with RT and may mitigate xerostomia by accelerating the recovery of salivary gland stem cells.

Level of evidence: Not applicable.

KEYWORDS

head and neck neoplasms, *Nigella sativa* oil, radiation injuries, salivary glands, thymoquinone, xerostomia

Marie Luff and Lauran Evans contributed equally to this study.

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1 | INTRODUCTION

Xerostomia, commonly known as dry mouth, is one of the most common complications during and after radiotherapy (RT) for head and neck malignancy.[1] It is approximated that 70% of patients develop xerostomia after receiving head and neck RT.[2] Patients experiencing xerostomia often report a lower quality of life, as this condition causes loss of taste, difficulty swallowing, and problems with speech; furthermore, xerostomia results in an increased risk for serious health consequences, such as increased dental caries, tooth decay, and risk for oral *Candida* infection.[3,4] Of all patients with xerostomia, those with a history of head and neck radiation have the lowest salivary flow rate and the worst reported symptoms.[4]

Radiation-induced xerostomia occurs due to terminal damage to both major and minor salivary glands in the oral cavity, or oropharynx. The major salivary glands include the parotid, submandibular, and sublingual glands (SLGs), which account for 90% of saliva production. The submandibular glands (SMGs) produce >60% of total daily saliva secretion and account for most unstimulated saliva volume, whereas the parotid glands contribute to most stimulated saliva volume.[5,6]

RT has been shown to result in significant and early loss of epithelial acinar cells, which are the predominant cells involved in saliva production, controlling both the volume and protein content in saliva.[2,5] The specific mechanisms resulting in acute and persistent xerostomia have not been elucidated and are hypothesized to be a result of macroscopic and microscopic changes to glandular tissue. Macroscopic changes include gross reduction of submandibular and parotid gland size and volume,[2,7,8] whereas microscopic changes include acinar cell loss and increase in apoptotic cell count, cytoplasmic vacuolation, hypovascularization, fibrosis, and interstitial edema.[2]

Salivary gland health is maintained by a small group of cells, called epithelial stem/progenitor cells, that possess the capacity to repopulate and differentiate into the needed cell types upon tissue injury; depletion of these stem/progenitor cells decreases salivary gland regeneration.[9] Although the characterization of stem/progenitor cells for the epithelial salivary glands are incomplete and evolving, and lineage-tracing for these cell populations constitutes an active area of research,[10,11] cytokeratin 5 (K5), an epithelial basal cell protein normally expressed in the basal-myoepithelial cell layer of the SMGs and other organs,[12] has been suggested to serve as a marker for multipotent cells with progenitor characteristics.[13] Ductal stem cells develop a salivary gland by differentiating into acinar or ductal progenitor cells. It is thought that acinar progenitors produce differentiated mucous and serous acini, whereas ductal progenitors produce ductal cells.[14] Thus, K5 ductal stem cell survival is thought to be important for repopulating a salivary gland after injury.

Nigella sativa oil (NSO), a widely used medicinal agent, has shown promise in prior studies with roles as an anti-inflammatory, antimicrobial, and antiemetic agent. The main chemical component found in NSO are quinines—in particular, thymoquinone (TQ), which constitutes 30%–48% of the oil.[15] Recent publications with NSO suggest that NSO supplementation may have hypoglycemic, hypolipidemia, and bronchodilatory effects, as well as a role in obesity management.[16,17] Other studies have shown that TQ has radioprotective effects on salivary glands (in an animal model) by free radical scavenging.[18]

The objective of our study was to determine if NSO can provide a protective effect on salivary gland stem/progenitor cells due to radiation damage. Furthermore, we aimed to assess if NSO would mitigate the development of xerostomia by protecting ductal stem cells that can repopulate to produce functional salivary glands after radiation-induced damage.

2 | MATERIALS AND METHODS

2.1 | Animal protocol

Forty, 10-week-old male C3H/HeJ mice (The Jackson Laboratories, Bar Harbor, ME, USA) were used in this study (Animal Research Committee [ARC], protocol number 2008-147). The Chancellor's Animal Research Committee of the University of California, Los Angeles, and the Animal Research: Reporting In Vivo Experiments (ARRIVE)[19] guidelines and protocols were approved and followed. Forty male C3H/HeJ mice were randomized to one of four experimental groups (Figure 1). The four experimental groups consisted of: sham RT with H_2O gavage ($N = 4$), RT with H_2O gavage ($N = 12$), sham RT with NSO gavage ($N = 12$), and RT with NSO gavage ($N = 12$) (Figure 1). After RT was administered on day zero of experimentation, mice were given a standard soft diet and weighed daily to ensure adequate nutritional intake and health.

2.2 | Ionizing radiation

Mice were irradiated on day zero of the experiment. Irradiation was performed by anesthetizing the mice with a ketamine hydrochloride (80 mg/mL)/xylazine hydrochloride (6 mg/mL) cocktail. A standard dose of 0.2 mL per 20 g mouse weight was administered.[20] Anesthesia was confirmed with toe pinch and a sterile protective ophthalmic ointment was applied to the eyes. Prior to RT, lead shielding was placed over the mice to expose only the cephalic area to the radiation field. A 15 Gray (Gy) single dose RT was chosen as previous literature demonstrated that a 13–15 Gy single dose of RT is sufficient to produce an 80% or greater loss in salivary function.[21,22]

2.3 | *Nigella sativa* oil (NSO) and thymoquinone (TQ) treatment

Treatment schemes varying the dosage of NSO and length of time of gavage were tested (data not shown). We observed that treatment of 3 days before followed by 15-day post RT was the best regime for NSO gavage treatment. Due to difficultly solubilizing NSO, intraperitoneal administration of NSO was not attempted. All treatment groups were gavaged starting 3 days before RT and continuing until 15 days post RT. NSO treatment groups received 0.07 mL/kg.

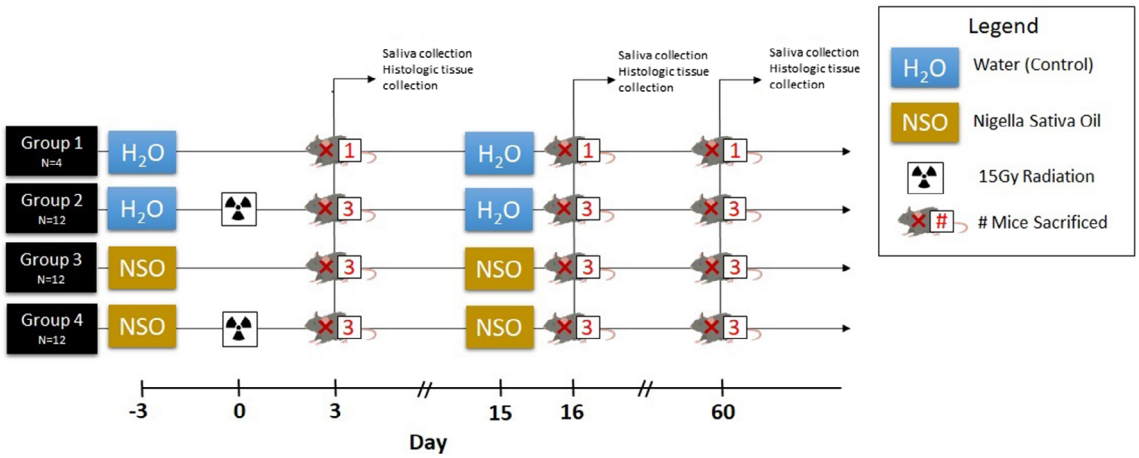

FIGURE 1 The four experimental groups consisted of: sham RT with H_2O gavage ($N = 4$), RT with H_2O gavage ($N = 12$), sham RT with NSO gavage ($N = 12$), and RT with NSO gavage ($N = 12$). RT (15 Gy dosage) was administered at day 0. One to three mice from each experimental group were sacrificed at the timepoints of days 3, 16, and 60. NSO, *Nigella sativa* oil; RT, radiotherapy.

Concentration of TQ in NSO can vary widely due to growth conditions and other environmental factors therefore dose was calculated on NSO gavaged.[23–26]

2.4 | Pilocarpine stimulation and saliva collection

On days 3, 16, and 60 of experimentation, mice were randomly selected from each experimental group for pilocarpine stimulation and saliva collection (Figure 1). A standard protocol for salivary functional assessment with pilocarpine stimulation was followed.[25] Using a 28G × ½ needle, mice were anesthetized with intraperitoneal injection sterile saline solution of 100 mg/kg ketamine and 10 mg/kg xylazine based of off mouse weight. A cotton-tipped applicator was used to gently apply lubrication to the eyes. Mice were kept on a warm surface whereas all following procedures were performed at room temperature. The nose, limbs, and tail were secured to a stage with surgical tape, and the neck was cleaned with an alcohol wipe. A superficial cut was made with dissecting scissors along the ventral midline of the neck, and scissors were used to separate the subcutaneous tissue planes. The incision was made 1 cm below the mouth. Two lateral incisions were made at the inferior and superior aspects of the first cut, and the skin was removed to reveal the structures of the head and neck. Using a dissection microscope, the SMGs were visualized and gently lifted with forceps, exposing the four infrahyoid strap muscles overlying the trachea. With dissecting scissors, the medial portion of the strap muscles were removed whereas remaining as midline as possible. Cuts were made only to visualize the trachea. Once the larynx, tracheal, and thyroid gland were visible, a horizontal incision was made in the trachea inferior/posterior to the thyroid using small dissecting scissors to ensure the airway was clear of fluid. For saliva collection, the dissection stage was first angled downward 45° cranially to assist with saliva flow. A 0.5 mL 28G × ½ needle was used to inject 10 µL/g body weight of pilocarpine, for a total dose of 100 mg/kg. The mouth was opened. Once a bead of saliva was observed in the mouth, the proximal end of a capillary tube was placed in the fluid, with the distal end placed into a collection tube. Excess saliva was collected by pipette and added to the collection tube. Saliva was collected for a total of 12 min after pilocarpine injections.

2.5 | Histologic analysis

Following pilocarpine stimulation and saliva collection, mice were euthanized by carbon dioxide asphyxiation. The submandibular, sublingual, parotid glands, and tongue were visualized and carefully dissected under a dissecting microscope at 8× magnification. All tissues were measured and weighed. Immediately following dissection tissues were preserved in PFA and paraffin embedded for histopathology slides. Histologic staining was performed simultaneously for specimens at each experimental timepoint. Each specimen was stained with standard hematoxylin and eosin (H&E), in addition to antibodies for inflammatory markers, including COX-2 (1:250), NF-kβ (1:200), TNF-α (1:200), and K5 (1:250). A standard immunohistochemical staining protocol and antibodies were used for staining (Abcam primary rabbit polyclonal antibodies ab15191 [10 µg/mL], ab16502 [1 µg/mL], ab6671 [10 µg/uL], ab53121 [10 µg/mL]; Abcam secondary goat anti-rabbit antibody ab205718 was used at 1:200). At experimental timepoints, days 3, 16, and 60, specimens from each of the four experimental groups were analyzed under 10× magnification. Inflammatory marker staining was quantified with ImageJ software.[27]

2.6 | Statistical analysis

Statistical analyses were performed using a Student's *t*-test for treatment group comparisons. Survival was analyzed via the Kaplan–Meier

method. The log-rank test was utilized to determine statistically significant survival differences, and a significance threshold of $p = .05$ was applied to the analysis.

3 | RESULTS

3.1 | Weight gain

Control mice (sham RT + H_2O gavage) displayed appropriate weight gain from days 0 to 30 of experimentation when compared to The Jackson Laboratory weight values[28] for healthy mice (Figure 2A). Compared to all other treatment groups, control mice had the expected weight gain at 60 days with an average final weight of 31.23 g and standard deviation of 0.832 g. Mice treated with RT + H_2O gavage did not recover their weight from days 0 to 30, and at day 60 were significantly underweight at 24.86 g ($p < .001$). Sham RT + NSO gavage and RT + NSO gavage mice initially lost weight after treatment but gradually gained weight over 30 days; however, they remained significantly underweight compared to the sham RT + H_2O gavage group at day 60 with an average weight of 27.1 g ($p < .001$) and 28.91 g ($p < .001$), respectively. However, both NSO gavage-treated groups gained significantly more weight than the RT-treated groups alone (Figure 2B).

3.2 | Survival

Both sham RT + NSO gavage and sham RT + H_2O gavage displayed 100% survival at day 60 of experimentation. Mice treated with RT + H_2O gavage had the worst survival rate of 75% at day 60, whereas mice treated with RT + NSO gavage had a better survival of 83% (Figure 2C).

3.3 | Salivary function

At day 3 of saliva collection, mice that received RT + H_2O gavage demonstrated significantly depressed salivary output (0.02 g) when compared to the control group (0.143 g, $p < .001$) and RT + NSO gavage treatment group (0.1217 g, $p < .001$). Mice treated with RT + NSO gavage had salivary output comparable to that of the control mice at days 3, 16, and 60. At day 60, all mice that survived had recovered salivary function regardless of their treatment arm (Figure 2D).

3.4 | Histologic analysis

H&E staining in RT + H_2O gavage and RT + NSO gavage revealed (Figure 3), that both submandibular and sublingual tissue had high

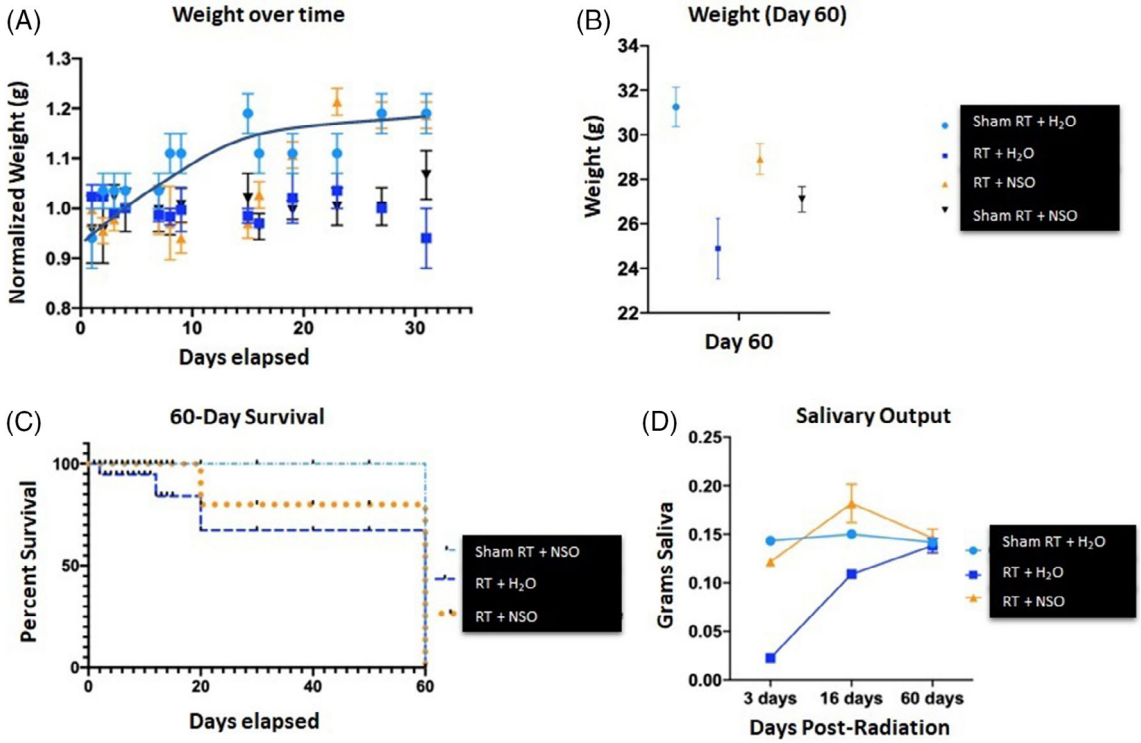

FIGURE 2 (A) The mouse weights (normalized to their starting weight) from day 0 to day 30 for each experimental group. The solid line represents the weight gain projected by Jackson Laboratories, representative of normal mouse weight gain. (B) Mouse weight in grams at day 30 for each experimental group. $N = 8$ for each experimental group. (C) Kaplan–Meier survival curve, independent of scheduled mouse sacrifices at days 3, 16, and 30. The light blue dotted line demonstrates that oral gavage with NSO does not impact survival. The dark blue line shows that 15 Gy radiotherapy does impact long term mice survival. Mice who received 15 Gy radiotherapy and NSO gavage fared better than mice who received 15 Gy radiotherapy and H_2O gavage. (D) Salivary output at days 3, 16, and 60 of experimentation. NSO, *Nigella sativa* oil.

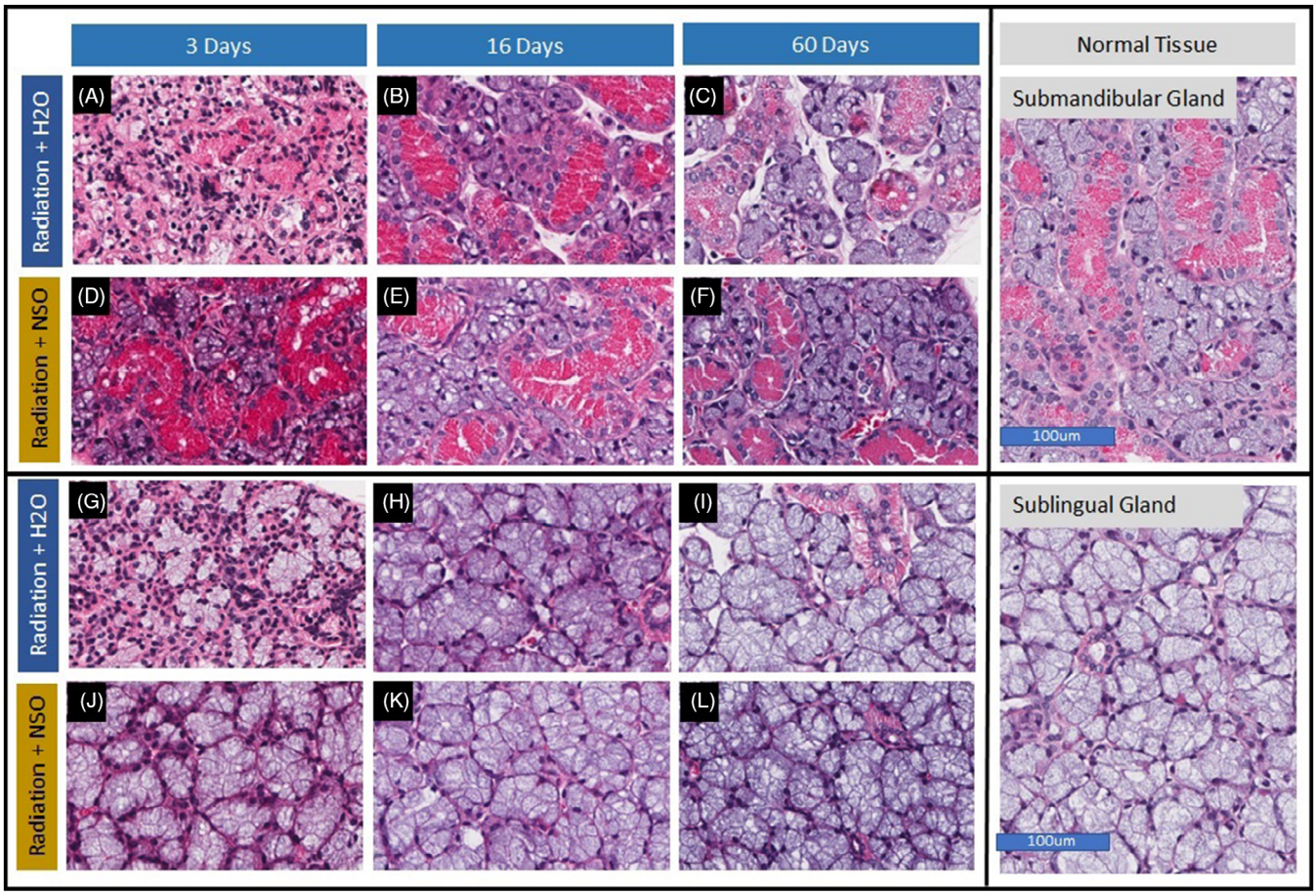

FIGURE 3 Submandibular gland (A–F) and sublingual gland (G–L) H&E-stained specimens at 40× magnification collected at 3, 16, and 60 days after 15 Gy radiation.

levels of eosin staining and dilated ductal cells at day 3. Day 60 post-RT, the submandibular tissues in the RT + NSO gavage had less porosity and tissue atrophy present when compared to RT + H_2O gavage, whereas H&E staining of the SLG displayed a similar phenotype as sublingual tissue. At day 60, the sublingual and submandibular tissues treated with RT + NSO gavage had morphology more similar to unirradiated tissues than the morphology of the RT + H_2O gavage group. As a control, tongue tissues were also stained with H&E and analyzed, however, no significant difference in levels of eosin stain or morphology was found between treatment groups.

When assessing staining for inflammatory cell markers, COX-2, TNF-α, and NF-kβ (Figure 4/Figure 5), the submandibular and sublingual tissues both demonstrated high COX-2 staining at 3 days post RT regardless of NSO treatment, with staining intensity gradually decreasing at 16 days with a further reduction in staining at day 60. SMG staining did not have any statistical difference at any timepoint between H_2O and NSO-treated groups, whereas data on the SLGs revealed that in RT + NSO gavage-treated mice, staining intensity returned to normal at day 16 with RT + H_2O gavage staining remaining elevated (RT + NSO gavage relative staining intensity of 47 au, RT + H_2O gavage relative staining intensity of 75 au, $p < .05$). COX-2 staining was equivalent at day 60 in the SLGs. K5 staining showed significant amounts of staining at 3 days both in the submandibular and in the sublingual areas in RT + NSO gavage and RT + H_2O gavage. At 16 days SMG staining again remained the same between both treatment groups whereas the SLG had more staining in the RT + NSO gavage group (RT + NSO gavage relative staining intensity 74 au, RT + H_2O gavage relative staining intensity 50 au, $p < .05$). At day 60, in both the SMG and the SLG, the RT + NSO gavage group showed stronger K5 staining ($p < .05$) when compared to the RT + H_2O gavage group (SMG: 84.5 au vs. 65 au, SLG: 145 au vs. 80.2 au).

4 | DISCUSSION

There are no current effective treatments for radiation-induced xerostomia. Several groups have employed surgical treatment options to preserve SMG function. A 2012 multicenter clinical trial demonstrated that SMG transfer prior to radiation therapy was 70% effective in the prevention of xerostomia.[29] However, surgical options typically present with more risk and longer patient recovery time. Temporary symptomatic relief can be offered by moistening agents and salivary substitutes for afflicted individuals; pilocarpine treatments of 5–

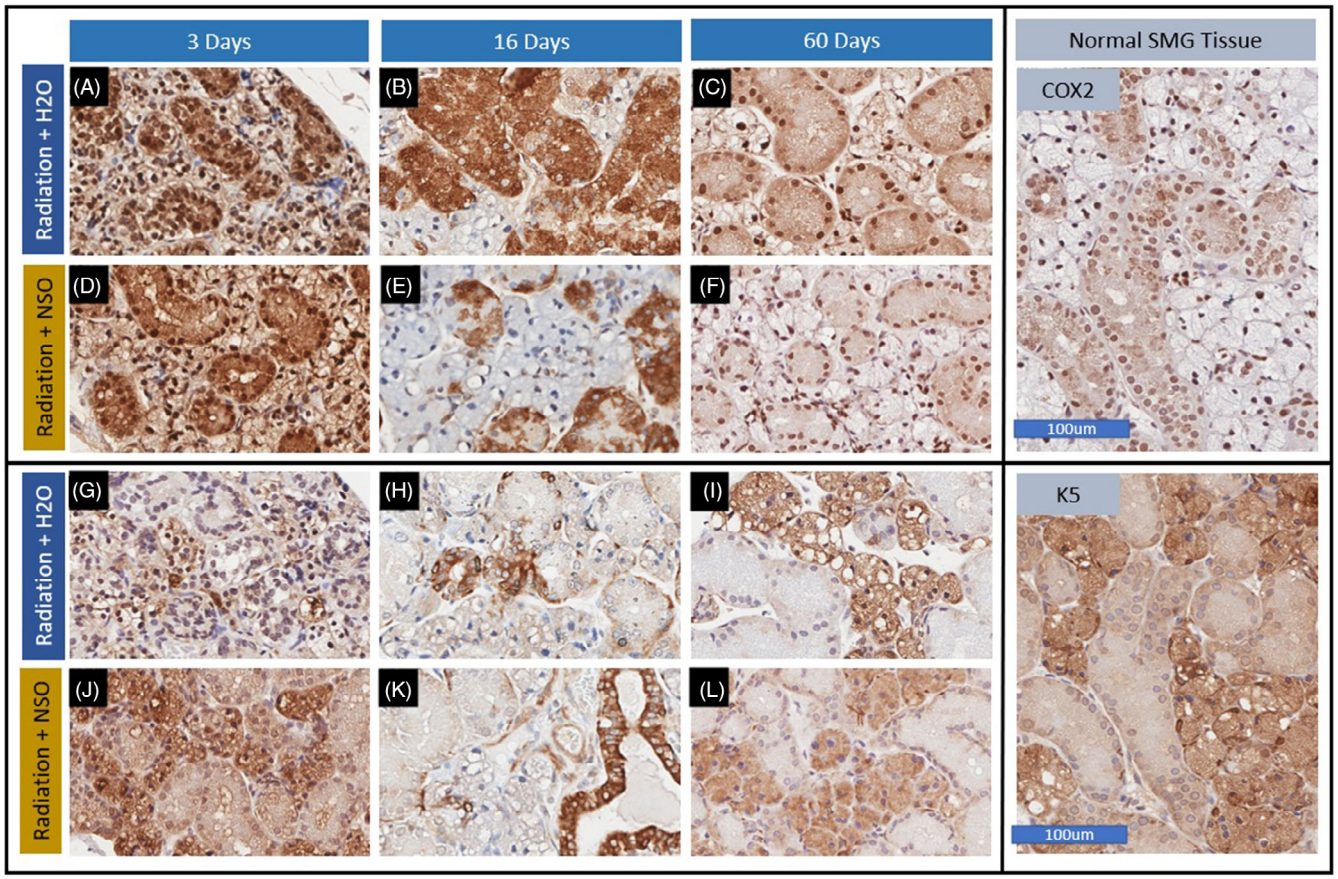

FIGURE 4 Submandibular gland (SMG) stained with COX-2 (A–F) and K5 (G–L) at 40× magnification. Specimens were collected at 3, 16, and 60 days after 15 Gy radiation.

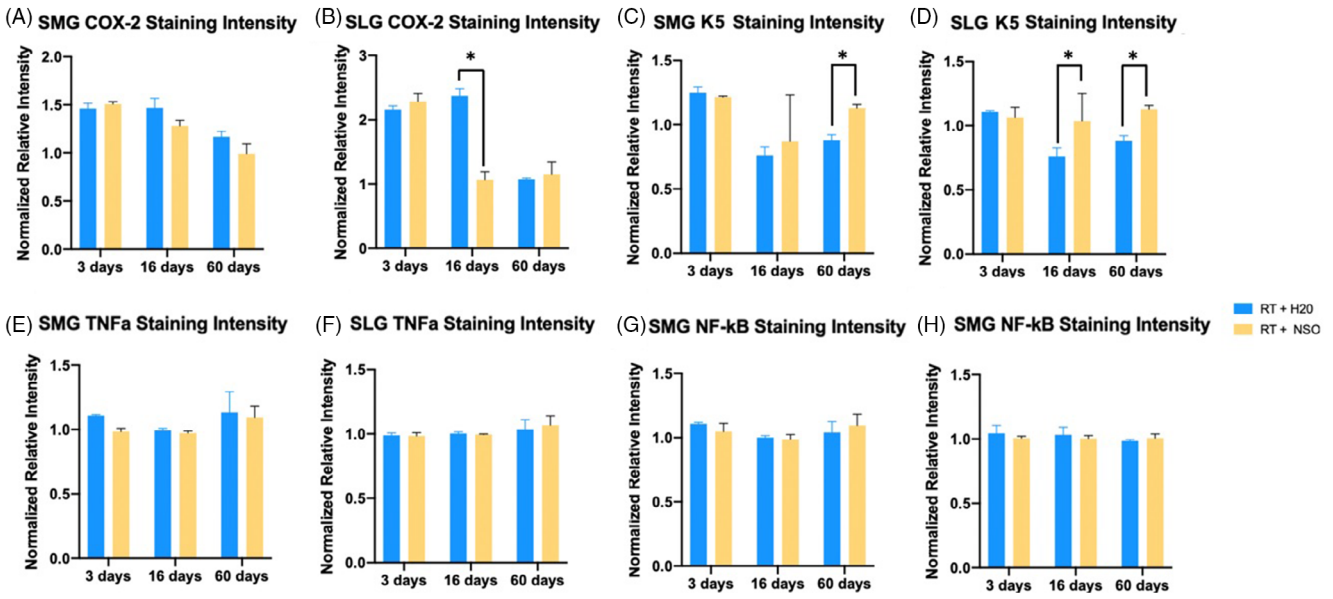

FIGURE 5 Inflammatory and stem cell staining quantification: (A and B) average COX-2 stain intensity for SMG and SLG tissues at days 3, 16, and 60. (C and D) Average K5 stain intensity for SMG and SLG tissues at days 3, 16, and 60. (E and F) TNF-α in SMG and SLG. (G and H) NF-kβ staining in SMG and SLG. SLG, sublingual gland; SMG, submandibular gland.

10 mg three times daily can be prescribed to patients with residual salivary function to improve salivary flow. Regardless, preventing atrophy and repairing salivary glands remains a clinical challenge.

NSO, and more specifically the active ingredient, TQ, provides a potential therapeutic avenue to prevent the loss of ductal stem cells during RT. Interestingly, TQ has also been shown to have anticancer properties whereas providing benefit to normal tissues.[30] Ductal stem cells give rise to all cell types within the salivary gland through two progenitor cell fate determinations: acinar and ductal. Ionizing radiation's primary mode of cell kill is via DNA damage either by directly or indirectly ionizing DNA through free radical formation, causing irreparable strand breaks. Protecting cells from radiation damage can occur by (1) increasing DNA repair capacity[31] or (2) reducing DNA damage received. It has been previously reported that TQ works as an antioxidant to slow free radical formation,[31] thus reducing DNA damage.

TQ reduces inflammation by modulating the expression of COX-2,[32] TNF-α,[33] and/NF-kβ,[34] at varying degrees. Our data herein demonstrates that the addition of NSO to RT-treated mice significantly decreases COX-2 expression at day 16 in the SLG as compared to mice not treated with NSO. However, there was no significant difference in COX-2 staining of the SMG between these groups at day 3, 16, or 60, or in the SLG at day 3 and day 60 (Figure 5). Inflammatory marker NF-kβ (Figure 5) showed no change in staining between NSO and H_2O gavage RT groups, whereas TNF-α (Figure 5) showed a marginal but nonsignificant change in staining across irradiated groups. Interestingly, TQ's ability to reduce COX-2 expression has been reported in the literature to be more potent than indomethacin, a prescription anti-inflammatory drug.[30] High COX-2 expression in cancer has been shown to induce cancer stem like activity in addition to promoting apoptotic resistance, proliferation, angiogenesis, invasion, and metastasis.[35] By inhibiting COX-2, it has been postulated that TQ (and subsequently NSO) may mitigate cancer progression and protect normal tissue. Whereas our results demonstrate that NSO may have some effect on COX-2 expression at day 16, our data did not illustrate evidence of COX-2 modulation via NSO at other timepoints. Salivary output collection demonstrated that NSO likely has the greatest protective effects on salivary gland function at days 3 and 16 after RT, with no difference in salivary output at day 60 between NSO treatment groups. In correlating our histologic inflammatory marker analysis with salivary outputs collected at identical timepoints, we may conclude that the most significant benefits of NSO on salivary gland protection occur acutely after RT.

Our data shows that acutely after radiation (day 3), NSO-treated mice have normal salivary output, whereas those that were not treated with NSO display a significant decrease in salivary output (Figure 2). This supports NSO's role in potentially contributing to a faster, reconstituted salivary gland. Although the mechanism of action of this faster reconstitution has yet to be elucidated, we postulate that NSO may have a role in protecting saliva-secreting acinar cells. Additionally, our data may support a role for NSO in protecting ductal stem cells. Histologically, K5 stained darker at all NSO-treated time points, except at 60 days, when compared to RT + H_2O gavage (Figure 4), which may indicate greater survival of ductal stem cells. Additionally, our data on day 16 demonstrates elevated levels of K5 staining for the NSO-treated groups as compared to the H_2O-treated groups, thus suggesting that NSO-treated groups may have recovered faster. Notably, our results show a significant difference between the RT + H_2O group and the RT + NSO group at day 16 in the SLG, but not the SMG, for both COX-2 and K5 staining. Whereas the mechanism contributing to this difference observed between these two glands is unclear, structural differences between the SLG and the SMG, such as the lack of intercalated or striated ducts in SLGs, may contribute to the significant effects of NSO observed on day 16.

NSO has been revered as a multipurpose medicinal herb and is used globally to treat ailments including liver concerns, infectious diseases, and wounds.[15] The safety profile of TQ has been shown to be favorable in mice, with a LD50 value >100 times the therapeutic dose used with oral gavage.[7,34,35] In humans, numerous randomized, double-blind, and placebo-controlled clinical trials have demonstrated that the ingestion and topical application of NSO has no serious adverse effects to the liver, kidney, or gastrointestinal system, as well as having no associations with adverse effects on platelet or total leukocyte counts.[16,36–38]

Although TQ has demonstrated an inherent ability to inhibit cancer cell proliferation in numerous cancers, including breast, colon, and lung cancer,[39] TQ has also been shown to augment the antitumorigenic effect of radiation on human head and neck squamous cell cancer cell lines,[40] thus decreasing concerns for tumor radioprotection and subsequent decreased survival if NSO is to be used to preserve salivary gland function in radiation-treated head and neck cancer. Research in this area remains limited, however, and the dual nature of TQ-selective radioprotection and its anticancer properties needs further exploration.

The strengths of our study include the simplicity of our experimental design, where we compare the outcomes of mice randomized to four distinct treatment arms over 63 days. All steps of experimentation, including mouse feeding, weighing, collection of salivary output, tissue collection, and histological staining were performed in parallel across all four treatment groups at identical timepoints, enhancing the accuracy and comparability of our results. Limitations of our study include small sample size ($n = 40$). Consideration must be given to the gavage process in mice, and any variability this may have caused in appetite, as well as translatability to human non-gavage administration. Additionally, whereas mouse and human salivary glands share many similarities, it is important to note that species-specific differences in drug metabolism, immune architecture, salivary gland anatomy, innervation systems, and regeneration mechanisms limit the clinical translatability of promising results seen in mouse models.[16,41,42] Future studies should include other progenitor/stem cells markers besides K5, such as SOX-2 and PIP, in addition to markers for cellular proliferation and apoptosis.

There is wide potential indicated for the therapeutic benefits of NSO and TQ on radiation-induced xerostomia in humans. Herein, we demonstrate promise for an NSO-based oral solution for prevention and treatment of RT-induced xerostomia. Because NSO is already

considered a widely used supplement, its translation into a clinical trial in patients undergoing head and neck radiation therapy is feasible.

5 | CONCLUSION

Our results demonstrate that the oral consumption of NSO, a widely used medicinal agent, may contribute to accelerated salivary gland function rescue within the first 60 days after RT in an animal model. Our findings support the potential use of oral NSO in patients who undergo RT to the head and neck as it is not only a widely used agent with minimal serious side effects, but it may also allow for the protection and recovery of salivary gland stem cells.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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