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Preemptive Pharmacologic Intervention in Radiation-Induced Salivary Dysfunction

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Abstract. **Xerostomia ("dry mouth") is a symptom of several diseases. It also occurs as a side effect of certain therapeutic interventions, most frequently pharmacotherapy. The most severe and irreversible forms of salivary dysfunction result from damage to or loss of salivary acinar cells. One of the severest forms of iatrogenic salivary gland destruction results from the therapeutic doses of irradiation given to treat head and neck cancer or to purge the bone marrow before transplantation. Xerostomia encompasses a wide range of involvement, from an inconvenience when mild, to a debilitating condition when severe. Reports of the symptom of dry mouth by patients do not always correlate with the degree of diminished salivary flow. However, a significant loss of stimulated flow makes it difficult to process solid food into a bolus that can be swallowed. If sustained, nutritional deficiencies may result. Saliva also facilitates formation of speech patterns. Its loss hinders speaking and communicating, possibly causing the patient to withdraw from social interaction. Together these conditions can impair the physiological and psychological well-being of the patient. Thousands of individuals undergo radiation therapy for head and neck cancer in the United States each year. Increasing numbers are receiving total body radiation before transplantation of bone marrow. Although the salivary gland is not one of the more actively dividing organs in the body, nevertheless it ranks as one of the most radiosensitive. The mechanism of this sensitivity is not understood. This article reviews human and animal pathophysiology of radiation-induced salivary damage. We also discuss animal studies that have employed various strategies to modify and clarify this process. Finally, we describe encouraging results from early clinical trials suggesting that protection of salivary glands during therapeutic irradiation may be possible.** [P.S.E.B.M. 1999, Vol 221]

Xerostomia is a subjective symptom of oral dryness that does not always correlate with actual salivary gland disease or flow rate, making diagnosis of the underlying cause difficult (1–5). Several excellent reviews of the general nature of this condition have been published (6–12). This review will focus on one of the more predictable causes of severe xerostomia.

Radiation therapy is a prevalent treatment modality for malignancies. In the United States several thousand individuals receive irradiation treatment for head and neck cancer each year. Inadvertent damage to the major salivary glands during treatment causes severe reduction in salivary output and xerostomia (13–17). The more recent innovation of bone marrow transplantation in oncology, with its associated total body irradiation, causes similar complications (18–21).

Of the factors that determine the severity of salivary gland damage caused by therapeutic irradiation, the two most important are the dose of radiation delivered and the volume of gland exposed to the radiation field (14, 17, 22, 23). Within the first week of radiotherapy, stimulated salivary flow decreases markedly (17). Symptomatic improve-

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ment of xerostomia may occur after several weeks, but flow rate measurements of whole saliva indicate flow rate falls and remains significantly depressed months and even years after irradiation (13, 16, 17, 24–28). At doses of less than 50 Gy, the depression of salivation may be partially reversible, but higher doses generally cause irreversible damage (14, 17, 29–31).

Reduction in salivary flow *per se* is not life threatening, but deterioration of dental and oral health has significant impact on the quality of life, and treatment is palliative at best (11, 12, 21, 32–41). The resulting mucositis and difficulty with mastication and swallowing can lead to nutritional deficiencies (42–46). Hyposalivation has also been shown to affect both subjective and objective measures of speech (47). Finally, the loss of the antibacterial properties of saliva can lead to opportunistic infections (40, 48).

Uniqueness of Salivary Gland to Radiation Damage

Although radiation-induced damage to salivary function is well known, the mechanism is poorly understood. DNA is a primary target for radiation damage, and tissues characterized by rapid cell division are generally most sensitive to early radiation damage. In these tissues (e.g., bone marrow, gastrointestinal epithelium, hair) critically damaged DNA leads to cell death after repeated attempts to divide (reproductive death). The parenchymal tissue of the major salivary glands, on the other hand, is characterized by postmitotic reverting cells that turn over slowly. Yet the parotid gland ranks as one of the more radiosensitive organs.

Attempts to explain this anomaly have led to extensive investigations for over 8 decades. These studies employed different sources, modalities and dosages of radiation; used different species; and measured different outcomes. This diversity makes synthesis and interpretation of the results of these studies difficult. Our objective in this review will be to summarize the consensus of understanding of radiation-induced salivary gland pathophysiology. We will review experimental studies that have employed strategies to protect the gland from damage. Finally we will discuss some of the early results of clinical approaches for protection.

Pathophysiology

Studies of many species, including humans, do agree that the intensity of salivary gland damage increases with radiation dose and exposed gland volume. One of the few studies of early radiation damage to human salivary glands described significant infiltration of the parotid gland by polymorphonuclear and eosinophilic leukocytes along with some plasma cells 24 hr after irradiation with 10–20 Gy (15). The acute inflammatory changes closely paralleled degenerative changes of the parenchymal tissue. These changes were most evident in the serous cells and included degranulation, cytoplasmic vacuoles, and pyknotic nuclei. The intercalated and interlobular ducts were dilated, but had intact epithelial linings. In the mixed submaxillary gland, similar acute inflammatory and degenerative changes were observed in serous cells, but little or no changes were seen in mucous cells.

Depending on dose and irradiation protocol (i.e., single versus fractionated doses) degeneration and loss of serous, and in some cases mucous, cells continues after irradiation (16, 49, 50). Although histopathological changes vary greatly among subjects, the glands atrophy, serous acini are reduced in number, and inflammatory cells can be observed during this time. After a year the changes appear to stabilize, and the glands generally become fibrotic, especially the submaxillary gland. Function, as measured by citric acid–stimulated uptake of 99 mTc-pertechnetate, declines with time over 3–36 months following 50–70 Gy irradiation depending on the volume of gland in the field (51).

Although more radioresistant than humans, the rat is the most frequently used animal model in the study of salivary gland damage by irradiation. Functional studies in the rat suggest a ceiling dose equivalent to acute administration of 15 Gy (52, 53). Parotid flow decreases as early as the first 24 hr after irradiation and in most studies reaches a maximal decrease by 3 days (52–57). When high acute doses are employed (15 Gy or greater), much of this initial decrease appears to result from decreased food and water intake due to radiation-induced transient irritation of oropharyngeal tissues (58). Partial recovery may occur after the initial acute effect, but stimulated flow rate remains significantly depressed for up to a year after irradiation (53, 54, 59).

Functional changes of the submandibular gland are not as clear. Vissink *et al.* (53–55) reported that flow rate decreased and lag time (the period between stimulation and flow) increased in both parotid and submandibular glands. Both glands showed a maximal response by 3 days postirradiation, and the temporal sequence of further changes was generally similar for both glands. On the other hand, Nagler *et al.* (52, 56, 57) found that although both submandibular and parotid gland weight decreased by Day 3 following single doses of 7.5 Gy or greater, submandibular flow was not significantly affected at this time. By 40 days after irradiation, flow was decreased in both parotid and submandibular glands and remained depressed in both glands 90 days postirradiation. By this time gland weight was decreased by 62% and 39%, respectively. Twelve months after irradiation with 10 Gy, pilocarpine-stimulated parotid and submandibular flow were decreased by 80% and 75%, respectively (59). A significant contribution by systemic effects could be excluded since salivary changes occurred in the absence of decreased body weight as generally seen with higher doses. These investigators concluded that when evaluated over a 3–12-month period, both glands show similar dose-related decreases in gland weight and flow.

As with functional change following irradiation, observable degenerative morphological change occurs within the first few days after irradiation. However, in a study of both morphology and function in rat submandibular gland following a single dose of 15 Gy, Vissink *et al.* (55) con-

cluded that morphological and functional changes are not always temporally correlated. The most striking early light microscopic and ultrastructural changes were degranulation of the convoluted granulated tubules, fibril-like condensations in the mucous granules, destruction of mitochondria, distension of the nuclear membrane, and distension of the rough endoplasmic reticulum cisternae. By the 10th day following irradiation, these changes were markedly reduced, and signs of recovery were observed. However, flow rate remained significantly depressed, and radiation-induced changes in Na^+ and K^+ were still present. They concluded that radiation-induced functional changes persist after morphology has regained a nearly normal appearance.

Savage *et al.* (60, 61) observed similar morphological changes in rat submandibular gland following irradiation with 5–10 Gy administered as a fractionation protocol of 2.5 Gy/dose/day. Three days following irradiation, they observed a marked decrease in the size of the acinar cells, signs of atrophy, and isolated cases of cellular structural change. The severity of these changes was dose-dependent. Fourteen days following irradiation, the glands showed significant recovery with increased vascularity; the greatest recovery seemed to occur after the highest dose. Functionally, uptake of radiolabeled leucine was increased compared to unirradiated controls.

In the rat parotid gland, acute doses of 16 and 64 Gy caused equal morphologic damage up to 2 days following irradiation (62, 63). After this period, the higher dose produced more extensive damage, described as focal cytoplasmic degeneration or as necrosis of whole cells. "Cytolytic bodies" contained remnants of endoplasmic reticulum, mitochondria, and secretory granules and were only visible with the electron microscope. Large areas of necrosis appeared as "light bodies" under the light microscope. Later observation showed signs of removal of cellular debris and acinar cell atrophy. Eight days after irradiation with 16 Gy the tissues looked reasonably normal; the small lobules of parenchyma and increased density of ducts suggested a great deal of tissue loss in the specimens from rats irradiated with 64 Gys. This was supported by a significant loss in gland weight (> 57% of control). Importantly, in both cases, the surviving acinar cells appeared normal. Changes in the ducts during the study were unremarkable.

In summary, morphological evidence from the rat demonstrates that, except for doses at which frank necrosis predominates, the intensity, but not the nature, of degenerative change increases with dose. Effects on acinar cells generally resemble those seen in humans. Serous cells show the most damage in terms of degranulation, loss of cells, and atrophy earlier and at lower doses. Mucous cells are less affected. Ducts show some minor changes, but generally retain their integrity. A period of repair follows, but evidence of lost cells remains.

Radiation-induced salivary gland damage has been studied in other species. At 4 and 10 months following irradiation with a single 15-Gy dose, rabbits gained less weight and had lighter submandibular glands as compared with nonirradiated control animals (64, 65). Histologic examination revealed evidence of partial atrophy, modified architecture, and decreased granular size in the seromucous acini. By 4 months, the serous tubules were degranulated, but significant regeneration had occurred by 10 months after irradiation.

Expecting a greater similarity to humans, Stephens *et al.* (66–69) studied morphological changes in primates after irradiation with doses ranging from 2.5–15 Gy. The number of degenerating and necrotic cells increased with both time and dose. With doses less than 7.5 Gy, necrosis involved only occasional individual cells whereas higher doses resulted in necrosis involving all cells comprising some acini. Changes in acinar cells were similar to those seen in other species (i.e., serous cells of both the parotid and submandibular glands were damaged by lower doses). After doses of 12.5 Gy or greater, mucous cells in the mixed submandibular gland were damaged but to a lesser extent than serous cells. Nuclear changes included progressive clumping of chromatin material beginning within the first 6 hr after irradiation and culminating by 24 hr in a marked increase in the number of serous cells with pyknotic or darkly stained, crescent-shaped nuclei. Beginning at 16 and up to 40 weeks after irradiation, atrophy due to loss of serous acini was the most striking feature observed.

Although many of the morphological changes appear similar across species, the presence of inflammatory infiltrate, so characteristic of human histologic specimens, varies depending on the species studied. Human histopathological studies suggest that inflammatory cell infiltrate is closely correlated with actual degenerative damage of serous cells (15). However, with few exceptions morphological studies of the rat report no significant inflammatory infiltrate (55, 60, 61). On the other hand, irradiation of the rabbit and primate was accompanied by the presence of inflammatory infiltrate in submandibular histologic specimens (64, 67, 69). In the latter species the intensity of the inflammatory response did not correlate well with other damage. Since similar serous cell lesions have been reported in human, monkey, and rat parotid glands (with or without the presence of inflammatory infiltration) inflammation in the parotid gland is likely a nonspecific radiation response and cannot explain all the degeneration observed.

Loss and Replenishment of Acinar Cells

As noted above, the salivary glands appear unique in their sensitivity to damage from irradiation. The relatively low mitotic activity of the salivary glands coupled with the rapid acute response suggest something other than reproductive death as the likely single explanation. Several laboratories have suggested that the earliest signs of dysfunction are due to early death of serous cells resulting from membrane disruption and interphase death (70–73).

Stephens *et al.* (67, 69, 74) concluded that acinar serous cell damage is the major event in radiation-induced injury of

rhesus monkey parotid gland. They speculated that serous cell loss is primarily due to interphase cell death and described two types based on morphology: necrosis at higher doses and apoptosis at lower doses. Other morphological studies point toward apoptosis as an important participant in the early death of serous cells of rat parotid and submandibular glands (60–62).

Later degeneration and atrophy are more consistent with reproductive death due to the slow turnover rate (70, 72). Savage *et al.* (61) proposed that the delayed, long-term decrease in cell populations following irradiation occurs when the normal cyclical loss of acinar cells cannot be replenished by a stem cell population depleted by apoptosis.

The studies cited above based their conclusions on observations of cells whose morphology was consistent with apoptosis. Confounding factors can make conclusive identification of apoptosis by morphology alone difficult (75). Studies that employ newer p53, caspase, and poly(ADP)ribose polymerase assays in combination with differential fluorescent staining techniques should further define the role of apoptosis in salivary gland damage.

Replenishment of lost ductal and acinar cells in rodent salivary glands has been suggested to occur by differentiation of progenitor cells in the intercalated duct (76, 77). Using ^{3}H-thymidine pulse labeling in the submandibular gland of male mice, Denny *et al.* (78) reported evidence from which they postulated that self-proliferation of cells may play a significant role in some compartments. Their data indicated that at the junction of intercalated and granular ducts, intercalated cells differentiated into granular duct cells, probably *via* striated granular duct cells. However, an analysis of total radiolabeled granular ductal cells led them to estimate that approximately 70% of the cell population is maintained by self-proliferation as opposed to 30% by differentiation from progenitor cells. They also concluded from a similar analysis of the intercalated duct-acinus junction, that self-proliferation accounts for most of the proliferative activity responsible for maintaining the acinar cell population. Similar results have been reported for the rat submandibular gland (79).

Consistent with this hypothesis, Peter *et al.* (80) reported that cell proliferation could be observed in the intercalated duct compartment of rat parotid and submandibular glands within 3 days after irradiation with 15 Gy as a single dose. Six days after irradiation, proliferative activity was observed in acinar, granular convoluted tubule, and striated duct cells. Their methods did not allow them to distinguish the source of the proliferating cells (i.e., progenitor cells from the intercalated ducts versus self-proliferation of cells within the other epithelial compartments of the glands).

The above studies are further supported by the observation that irradiation increases the expression of the early response genes, *jun*B, c-*jun* and c-*fos*, whose regulation is associated with DNA damage (81). These genes belong to a family of transcription factors that influence the transcription of cellular target genes. Mertz *et al.* (82) found that, in the rat parotid, expression of all three genes was increased within hours after irradiation with a single 15-Gy dose. In the rat submandibular gland, expression of *jun*B was increased by irradiation, but tissue-specific genes associated with routine function, such as those for kallikrein, proline rich proteins, and ubiquitin, were unaffected (83). The c-*fos* oncogene was also unaffected.

In Vivo Versus *In Vitro* Response

Most of the studies that examined the effects of irradiation on salivary glands involved irradiation and assessment of response *in vivo*. Bodner *et al.* (84) reported an interesting study demonstrating that although *in vivo* salivary function was damaged following irradiation, cellular function of surviving acinar cells did not appear to be affected when tested *in vitro*. Rats were irradiated with a single 20-Gy dose, and both *in vitro* and *in vivo* parotid function was evaluated 1, 3, and 7 days after irradiation. Body weight and *in vivo* pilocarpine-stimulated parotid flow rate declined markedly over the 7-day period of observation, and the microscopic morphology was similar to that described above.

In contrast, processes related to exocrine function were unimpaired when assessed *in vitro* using dispersed acinar cells. Amylase release stimulated by isoproterenol, although depressed 1 day after irradiation, was no different from control cells by the third day. Epinephrine-stimulated K^+ efflux was not affected at any of the time points measured. Carrier-mediated uptake was assessed by measuring α-aminoisobutyric acid uptake on Day 3 postirradiation and was similar to control cells. Energy utilization was also assessed 3 days after irradiation by measuring glucose oxidation and was unaffected. Their results suggest that acinar cells that survive irradiation are functionally similar to unirradiated cells. The plasma membrane appeared to be intact and functioning. Membrane receptors, α- and β-adrenergic, were functional, as were the signal transduction pathways leading to K^+ and amylase release.

Similar results were reported in later studies by Franzén *et al.* (85–87) who studied the early and late *in vitro* cellular responses of rat parotid acinar cells following *in vivo* fractionated irradiation. Dose fractions ranged from 4–9 Gy administered daily for 5 days, and function was assessed at 10 and 180 days following irradiation. At total doses comparable to the 20 Gy employed by Bodner *et al.* they reported norepinephrine stimulated potassium secretion (as measured by $^{86}Rb^+$ efflux) to be unaffected. Potassium secretion was depressed in a dose-dependent manner at the higher doses employed in these studies. Norepinephrine-stimulated amylase secretion (exocytosis) was unaffected except at the highest dose (45 Gy) studied. This dose also increased release of unstimulated amylase compared to cells from unirradiated control animals.

Similarly, we found that single-dose irradiation (15 Gy) *in vivo* had no effect on isoproterenol-stimulated mucin secretion by rat submandibular gland fragments *in vitro* mea-

sured over a 1000-fold dose range (88). Although mean gland weight was reduced, uptake of [^{14}C]glucosamine was not affected, suggesting that surviving acinar cells retained the ability to take up glucosamine, incorporate it into mucin, and secrete the labeled product.

These studies suggest the possibility that at least some of the early functional deficiency following salivary gland irradiation may result from effects external to the acinus cell itself. The demonstration that uptake of α-amino[^{14}C]isobutyric acid into parotid acinar cells is impaired *in vivo*, but not *in vitro*, following irradiation suggests that impairment of transport of substances from the plasma to the gland may be involved (89). However, most animal studies provide little evidence for radiation induced impairment of the salivary gland microcirculation.

An early morphological study of the microvasculature of rat parotid gland following doses of 16 and 64 Gy revealed the usual changes in acinar cells but no significant morphological changes in the vessels of the microcirculation (63). Permeability was also unaffected as measured by lack of leakage of colloidal carbon or trypan blue. Later light and electron microscopic studies described a similar lack of significant effect of irradiation on the morphology of vessels of the microcirculation of rat, monkey and rabbit salivary glands (60, 61, 64, 67, 68, 90).

Hiramatsu *et al.* (91) measured blood flow and blood-to-tissue partition coefficients of the three major salivary glands in rat 3 days after irradiation with a single 15-Gy dose. In addition to controls fed *ad libitum*, they included pair-fed controls to control for the oropharyngeal syndrome commonly experienced after irradiation. Following irradiation, the blood-to-tissue partition coefficient, but not blood flow, was significantly depressed compared to the *ad libitum* control animals. However, both functions were more depressed in the pair-fed controls than either the irradiated or the *ad libitum* controls. These investigators concluded that their data agreed with early morphological studies suggesting that early after irradiation, the microvasculature supplying the salivary glands is not significantly affected. A study of blood flow through irradiated rabbit submandibular gland 4 and 10 months after irradiation engendered a similar conclusion (92).

Interruption of neural stimulation of the salivary glands provides another possible explanation for the difference between *in vivo* and *in vitro* acini function. This possibility was not supported by a study of adrenergic nerve function in rat parotid gland 3 days following a single 20-Gy dose of irradiation (93). These investigators employed the conversion of [^{3}H]dopamine to [^{3}H]norepinephrine to examine the ability of the adrenergic nerves to take up the catecholamine, translocate it to the vesicles, and convert it to the neurotransmitter.

Following irradiation, *in vivo* parotid and whole saliva flow was determined in response to injected norepinephrine and pilocarpine, respectively. Irradiation reduced pilocarpine-stimulated whole saliva flow to 50% of that of control animals. Although norepinephrine-stimulated parotid flow was completely inhibited following irradiation, uptake and conversion of [^{3}H]dopamine were unaffected in the parotid or submandibular gland as well as the cardiac left ventricle. These investigators concluded that irradiation eradicates parotid acinar cell responsiveness to norepinephrine without affecting parotid sympathetic nerve function. Release of formed neurotransmitter was not studied.

To our knowledge no one has performed similar studies of parasympathetic function following irradiation. Forsgren *et al.* (94) did report an increase in immunoreactivity of substance-P, a neurotransmitter involved in parasympathetic nerve-evoked salivation, in ganglia and nerve fibers associated with submandibular acini and small ducts following bilateral fractionated irradiation.

In summary, the studies discussed above evoke an interesting, but unexplained, incongruity. On the one hand, irradiation with doses in the range of 20–25 Gy decreased the *in vivo* rat parotid secretory response to norepinephrine or pilocarpine injected intravenously. On the other hand, parotid acinar cells from irradiated rats responded normally to norepinephrine's stimulation of potassium secretion *in vitro*. Activation of membrane potassium channels triggered by the increase in cytoplasmic calcium following activation of membrane receptors by released neurotransmitter is thought to be a key mediator of primary saliva formation (95, 96). Irradiation does not affect intracellular calcium release or extracellular calcium entry in transformed human submandibular ductal cells, or in acinar or ductal cells of rat parotid or submandibular gland (97, 98). Response to a circulating secretogogue such as norepinephrine or pilocarpine would not be expected to be affected by damage to nerves innervating the glands. One possible explanation for the above incongruity would be a defect in distribution of the secretogogue from the plasma to its site of action (i.e., membrane receptors on the acinar cell). However, the functional studies cited above provide conflicting evidence regarding this possibility (89, 91). Further studies designed to determine quantitatively the distribution of norepinephrine or pilocarpine to the acini should enhance our understanding.

Fractionated Dosing

Presently, therapeutic administration of irradiation is usually spread over several days to minimize damage to healthy tissues and organs. Several animal studies have evaluated the effects of fractionation protocols. In general, the results of these studies suggest that although damage can be delayed in some instances, salivary gland dysfunction still occurs in a dose-dependent fashion.

As discussed above Franzén *et al.* (85–87) found that the only functional change in rat parotid cells *in vitro* after fractionated irradiation was a dose-related decrease in potassium efflux. They observed no obvious morphological change 10 days postirradiation. A dose-dependent decrease in acinar cells was noted 180 days postirradiation, but oth-

erwise the morphology was similar to that of the contralateral control glands. The same dosage protocol (i.e., 4, 5, 6, 7 or 8 Gy/day for 5 consecutive days) reduced the whole saliva flow rate over a 26-week period after irradiation (99). Each dose reduced salivation after 26 weeks, but their data suggest that as the dose increases, functional damage appears earlier. One week following fractionated irradiation (8.6 Gy/day for 3 days), the rat submandibular gland exhibited almost complete destruction of granules with some recovery by 1 month (100). Ultrastructural damage exhibited a similar temporal sequence.

In one of the few direct comparisons between single and fractionated dose protocols, Vissink *et al.* compared the effects of 7.5 and 15 Gy given as single doses to two doses of 7.5 Gy each given 2, 3, 6, 12, 24, and 168 hr apart on *in vivo* rat salivary gland function (72). In general the changes in salivary lag time, flow rate, Na^+, and K^+, over the 30-day observation period were similar for the submandibular and parotid glands, although less severe in the former. Splitting the dose produced a dose-sparing effect on the lag phase and flow rate for both the early (<6 days) and late (>6 days) effects in both glands. Dose-sparing was apparent on salivary electrolytes and amylase only for the later effects.

Expressed as the single dose equivalent (i.e., the single dose required to produce a similar effect) dose-sparing was maximal at the 3- and 6-hr intervals and was equivalent to one-half the single dose. Increasing the interval for more than 6 hr generally decreased the dose-sparing effect. Morphologically, the mean granular content of the parotid acini and submandibular convoluted granulated tubules dropped dramatically within the first 2 hr following a single 7.5-Gy dose (equivalent to the first split-dose fraction). Regranulation began quickly and was back to control levels within 24 hr. This temporal inverse correlation between granular content and dose-sparing as the dose-interval increased may be particularly important in light of the suspected role of secretory granules in irradiation-induced damage (discussed below).

This same group investigated the effect of dose rate on radiation-induced changes in rat parotid and submandibular gland function (73). Total body irradiation with 7.5, 10, or 12.5 Gy was delivered at two different dose rates and followed by synergistic bone marrow rescue. The results confirmed that radiation-induced changes were dependent on the total dose delivered for both low- and high-rate administration. However, the maximum increase in lag time and decrease in flow rate of both glands occurred earlier and recovered more with the low-dose rate as compared to the high-dose rate.

Pharmacological Protection

Animal studies strongly suggest that the cellular and functional salivary gland damage induced by irradiation can be diminished by specific pharmacological agents. The drugs that have been shown to protect the salivary gland from radiation-induced damage belong to diverse pharmacological groups. Several possible mechanisms have been suggested to explain this protection: (1) attenuation of free radical damage; (2) stimulation of proliferation and repair; (3) modulation of signal transduction pathways; and (4) change of the oxygen level of the gland.

Figure 1 illustrates protection of parotid flow rate in male Sprague-Dawley rats by pretreatment with combined cholinergic and adrenergic agonists. Paired rats were randomly divided into two groups, and pilocarpine-stimulated parotid flow rate was measured. Three days later, the experimental group was given 0.6 mg/kg of carbachol (CARB), followed 15 min later by isoproterenol (0.2 mg/kg; ISO), both given by subcutaneous injection; both groups were then irradiated with 10 Gy. This procedure was repeated the next day (20 Gy total dose). Three-minute parotid saliva samples ($n = 4$) were collected on the days indicated. Change in flow rate was plotted on the ordinate as the decrease from the preirradiation rate (μl/min).

Generation of reactive oxygen species (or other free radicals) has been suggested as the initiating event in indirect irradiation-induced tissue damage, and free radicals have been estimated to play a key role in up to two-thirds of the indirect cytotoxic effects of ionizing radiation (101–103). These highly reactive species initiate biological damage through different, time-dependent mechanisms that include DNA strand breaks, lipid peroxidation, and indiscriminate oxidation of organic molecules (104). Presumably the damage may occur to both parenchymal and nonparenchymal cells.

The suspected role of free radicals in radiation-induced tissue damage has prompted studies directed toward the potential protective effects of free-radical scavengers. Arima and Shiba demonstrated a protective effect of glutathione in irradiated rat parotid gland (105). Intravenous administration of catalase, which limits formation of hydroxyl radicals from hydrogen peroxide, caused a consistent reduction of radiation-induced morphologic abnormalities in both the vascular endothelium and squamous epithelium of the rat (102).

Sodicoff and Conger compared protection of the rat

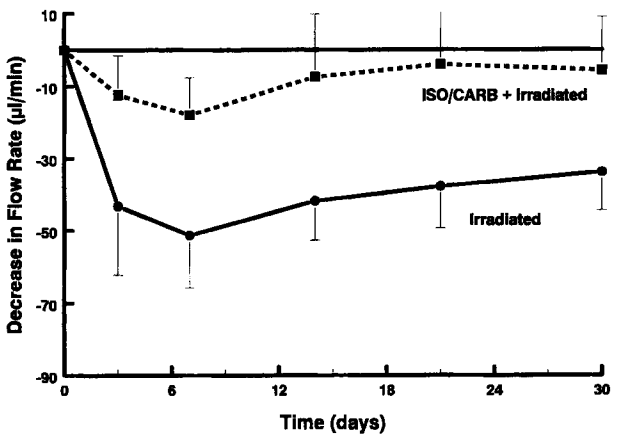

Figure 1. Taylor SE, Al-Hashimi I, Hashem R (unpublished).

parotid gland from radiation-induced atrophy by acute administration of isoproterenol and the aminothiol free radical scavenger, WR-2721 (106, 107). Both agents conferred protection, but they differed in that propranolol blocked protection provided by isoproterenol but not WR-2721. Interestingly, isoproterenol elevates the ratio of reduced/oxidized glutathione in rat parotid gland (108).

Other laboratories studied the radioprotectant effects of WR-2721 in the form of amifostine in animal models (106, 107, 109–113). The FDA has approved this drug to reduce cumulative renal toxicity associated with repeated administration of cisplatin. Amifostine, a pro-drug, is dephosphorylated by membrane-bound alkaline phosphatase to a pharmacologically active free thiol metabolite, WR-1065, which protects normal tissue from radiation damage while leaving tumors sensitive (114). Although its exact mechanism of cytoprotective action is unknown, a major part of its radioprotectant effect is believed to be due to free radical scavenging (103, 113). Amifostine effectively distributes to the salivary gland and provides a two-fold resistance to irradiation as measured by the dose reduction factor (115, 116).

As discussed above, numerous studies have established that serous cells are more radiosensitive than mucous cells. In 1984, Abok *et al.* (117) published an important paper in which they postulated that the granules within the serous cells may increase radiation-induced salivary gland damage *via* potentiation of free radical formation. Free radical damage of the granule membrane, presumably by lipid peroxidation, could in turn allow leakage of the proteolytic enzymes contained within the granules into the cell cytoplasm.

Using norepinephrine and phenylephrine to decrease, and atropine to increase, the granular content of serous cells, these investigators demonstrated a positive correlation between the granular content and radiosensitivity of serous intralobular cells of rat submandibular gland, as measured by morphological changes and cell loss. Using sulfide silver staining and particle-induced X-ray emission spectroscopy analysis, they also demonstrated that the serous, but not mucous or striated ductal cells, contain granules that in turn contain heavy metal ions, and that the granular content of these ions was increased by atropine. The most prominent transition ion was zinc, but the granules also contained ions of iron and manganese.

Heavy metal ions such as iron are known to catalyze formation of reactive oxygen species according to the following reactions in which $O_2^{-\cdot}$ and $\cdot OH$ are the superoxide and hydroxyl free radicals, respectively (104).

$$O_2^{-\cdot} + Fe^{+3} \rightarrow O_2 + Fe^{+2}$$

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + \cdot OH + OH^-$$

$$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + \cdot OH + OH^-$$

The above report stimulated a number of studies designed to explore the relation between granular content and radiosensitivity. The salivary glands respond to both divisions of the autonomic nervous system, and numerous drugs have been used to modify the salivary granular content. In a study of autonomic agonists, the β-adrenergic agonist, isoproterenol, was reported to degranulate rat parotid serous acinar cells completely (118). In the submandibular gland, it produced degranulation of 80% of the mucous acinar cells but had no effect on the serous cells of the granular convoluted tubules. In contrast, the α-adrenergic agonist, phenylephrine, had little effect on the acinar cells of either the parotid or submandibular gland, but caused variable degranulation of the granular convoluted tubular serous cells. Degranulation induced by the muscarinic-cholinergic drug, methacholine, although stimulating watery saliva flow, was relatively unremarkable and incomplete.

The antitumor drug, cyclocytidine, which appears to have both α_1- and β-adrenergic activity, has been reported to be most effective in degranulating rat submandibular serous cells while producing only minimal ultrastructural changes in mucous cells (119, 120). Degranulation is complete 1 hr following intraperitoneal administration of the drug (121). Regranulation begins within 6 hr and is complete by 24 hr after administration.

Numerous investigators have used these and other drugs to degranulate the salivary serous cells and thus indirectly study the effect of reducing the content of heavy metal ions on irradiation induced damage. Degranulation induced by cyclocytidine was reported to be associated with a reduction of the degenerative morphologic and immunocytochemical changes in rat submandibular gland observed after both single and fractionated dose irradiation (100, 122–124). Lotz *et al.* reported that the parotid and submandibular glands from miniature pigs pretreated with a combination of metaproterenol (a β_2-adrenergic agonist) and carbachol (a muscarinic-cholinergic agonist) immediately prior to irradiation delivered in fractionated doses exhibited significantly fewer degenerative ultrastructural changes than glands from untreated controls (125).

A similar protective effect was reported in the rat submandibular gland for the muscarinic-cholinergic agonist, pilocarpine, when given alone 1 hr prior to single-dose, 18-Gy irradiation (126). Salivation became copious soon after intraperitoneal injection, and glands examined by electron microscopy 30 min later exhibited a significant reduction in the number of granular cells per gland as well as the granules per cell, although degranulation was incomplete. Compared to untreated, irradiated controls, the glands from the pilocarpine treated animals exhibited much less ultrastructural damage at 1 week when morphological changes were greatest in the controls. Four weeks after irradiation, the glands from the pilocarpine-treated animals appeared normal except for mild fibrosis.

Conversely, inhibition of secretion by atropine increased the granular content of the rat submaxillary gland and intensified radiation-induced degenerative morphologic changes (117, 126). Norberg *et al.* (127) examined the effect of obstructing the rat submandibular gland excretory duct

on irradiation-induced damage. Ligation alone precipitated disappearance of serous granules followed by loss of serous cells and shrinkage of mucous cells over the 4-week observation period. Irradiation alone, with a fractionated dose comparable to doses used to treat human head and neck cancer, caused degranulation and loss of serous cells as early as 4 days after irradiation followed by regranulation of surviving serous cells by 4 weeks. The combination of ligation and irradiation was reported to intensify the changes induced by ligation alone with complete loss of serous cells, disfigured and shriveled mucous cells, fibrosis, and marked inflammatory cell infiltrate.

In contrast to the above studies, pilocarpine has been reported to increase, and the weak anticholinergic agent, biperiden, to decrease, morphological damage in the rabbit submandibular gland following irradiation (65). The investigators attributed this disparate result to the fact that, unlike previous studies, they administered the drugs so that stimulation or inhibition of gland activity was maximal at the time of irradiation, possibly affecting the oxygen status of the gland. However, this work was flawed by lack of proper controls.

The above studies dealt solely with morphology. Nagler *et al.* (70, 128) extended the investigation of the connection between granular metal content and irradiation induced damage by evaluating the interaction between irradiation (single dose, 15 Gy), and the function, ultrastructural morphology, and iron and copper content of rat parotid and submandibular glands. They also measured saliva redox activity since only the redox-active fraction of iron and copper would be expected to catalyze formation of reactive oxygen species.

The only protective effect–as measured by attenuation of the radiation-induced decrease of the pilocarpine-stimulated flow rate–occurred in the parotid gland 2 months after irradiation following pretreatment with cyclocytidine. Neither cyclocytidine nor pilocarpine exhibited any protective effect in the submandibular gland. Morphological examination was consistent with the functional changes. Serous cells of parotid glands from animals pretreated with cyclocytidine were almost totally degranulated whereas pilocarpine pretreatment produced only marginal degranulation. Neither drug caused morphological changes of submandibular mucous cells.

Both the parotid and submandibular gland contained high iron and copper contents. Cyclocytidine significantly increased the iron and copper content of the pilocarpine-stimulated parotid saliva along with its redox activity as measured by DNA degradation. Although the submandibular gland contained similar amounts of iron and copper, cyclocytidine did not increase their content in submandibular saliva, nor did the saliva exhibit redox activity. Recently this same group has reported that the iron chelator, deferoxamine (as zinc-desferrioxamine), partially protected the parotid gland from radiation as measured by a decreased flow rate (129).

The discrepancy between cyclocytidine's protective effect in the parotid versus the submandibular gland may be explained by the parotid gland's greater proportion of granule-containing serous cells (70). In contrast, the serous cells of the granulated convoluted tubules make up a relatively small proportion of the submandibular gland. Consequently, although morphological studies like those discussed above demonstrate some protective action of cyclocytidine on the submandibular gland, protection of the small proportion of serous cells but not the more numerous mucous cells, cannot preserve the functional capacity of the gland.

At least one group of investigators has challenged the idea that heavy metal-containing granules play a major factor in radiation-induced salivary damage. Part of their argument is based on their morphologic observation that although pretreatment with isoproterenol caused complete degranulation by 3 hr, it did not prevent induction of nuclear aberrations or the subsequent lysis caused by irradiation in either parotid serous or submandibular mucous acinar cells during the first few days after irradiation (130). However, recovery was noticeably accelerated in parotid glands from the isoproterenol pretreated animals compared to those from irradiated, saline-pretreated controls. In a separate study, they reported results similar to that of Nagler *et al.* with cyclocytidine (discussed above) in that pretreatment with isoproterenol significantly attenuated the irradiation-induced decrease of parotid, but not submandibular, flow rate over the 10-day observation period (131). They also found that isoproterenol pretreatment did not prevent the increase in lag time of salivation onset caused by irradiation.

This same group recently published the results of a more extensive study of the effect of pretreatment with muscarinic-cholinergic, α-adrenergic, and β-adrenergic agonists, alone and in combination, on radiation-induced functional changes in lag time, flow rate, and salivary potassium, sodium, and amylase (132, 133). Their results failed to show a clear correlation between degranulation and protective effect. In addition, those drugs that did provide some protection were most effective in reducing late (i.e., greater than 3 days postirradiation) effects, indicating that protection was due to recovery of function rather than protection from initial damage. They concluded that degranulation may be coincidental, and that the mechanism of protection may be related to increased proliferation and regeneration via modification of signal transduction pathway(s)–either the phospholipase C/phophoinositol (muscarinic-cholinergic and α-adrenergic agonists) or the adenylate cyclase/cAMP (β-adrenergic agonists) pathway.

The observation that chronic exposure to isoproterenol produces hyperplasia and hypertrophy of salivary glands supports this hypothesis (134, 135). The majority of the early growth phase stimulated by chronic exposure to isoproterenol in rat submandibular and parotid glands appears to be due mainly to hypertrophy of acinar cells accompanied by cell division (136). About 50% of this "adaptive growth" is estimated to be radioresistant with the hyperplastic com-

ponent being radiosensitive. Termination of isoproterenol exposure reverses the process resulting in a loss of gland weight, protein, RNA, and DNA. This "regression phase" appears to be radioresistant.

One of the earliest studies of the effect of isoproterenol pretreatment on radiation-induced salivary gland damage was that of Schneyer *et al.* (137). They found that administration of isoproterenol for 2 or 3 days prior to irradiation protected against atrophy of the rat parotid gland, but that pretreatment for shorter or longer periods produced little or no protection. They concluded that protection from loss of weight occurs during the period when hyperplasia and polyploidy are maximal. Since a single acute administration of isoproterenol failed to protect the gland, they excluded stimulation of secretory activity as being involved. In an attempt to define the mechanism of this observation, Hall studied isoproterenol's radioprotectant effect in relation to varying rates of cell division and found that isoproterenol exerted its peak protectant effect when the gland was irradiated 21 hr after injection of isoproterenol (138). He proposed that this time coincided with the S phase of the cell cycle in the parotid gland.

In the rat parotid gland, acute administration of isoproterenol has been reported to provide protection from radiation-induced gland atrophy equal to that provided by the free radical scavenger, WR-2721, for both the acute (0–10 days) and chronic (60 days) phases (106). This protection is blocked by propranolol (107). Furthermore, cAMP, the second messenger produced as a result of activation of β-adrenergic receptors, has been shown to exhibit a radioprotective effect in the rat parotid gland, especially for the late chronic phase (60–90 days postirradiation) (112, 139).

These studies all measured gland weight as an index of radiation damage. As mentioned above, morphological changes do not always predict changes in function. This is illustrated in a study by Mertz *et al.* (82) who reported that single-dose (15 Gy) irradiation decreased rat parotid gland weight and saliva output. Although postirradiation administration of isoproterenol reversed weight loss, it had no effect on the diminished saliva output.

Taken together, the studies discussed above, as well as others, amply demonstrate that pharmacological agents can modify the salivary gland's response to irradiation, but our understanding of the mechanisms involved is far from complete. The lack of consistent correlation between changes in morphology vs. function makes synthesis of the findings resulting from these studies difficult. They are also generally limited by their lack of exploration of the dose–response relationship between drug and protection. One consistent finding seems to be that the pharmacological protection may be more profound in the parotid than the submandibular gland.

Although some evidence suggests that heavy metal–containing granules may not be essential for irradiation-induced damage, their role as potentiators has not been ruled out. Free radicals formed during irradiation have extremely short half-lives suggesting that their role is limited to causing very early damage, but they may also initiate sequential processes that become important after free radical formation has ceased (103). This suggests that more sophisticated experimental designs are needed to rule out completely or to quantitate the contribution of the granules.

The rational use of drug interaction comprises a likely fruitful line of investigation that has received only limited attention to date. The importance of exploring potential benefits of drug interaction is suggested by the clinical successes in the pharmacotherapy of such diseases as hypertension and cancer by combining agents with different mechanisms of action (140). Based on the aforementioned studies and the putative factors involved in irradiation-induced cellular injury and recovery, four, and possibly five, groups of agents appear to be potentially productive to screen initially: secretogogues (which stimulate oxygen utilization and secretory activity), degranulating agents, free-radical scavengers, and agents known to stimulate cell proliferation. Since the inflammatory response seems to be important in the chronic phases of salivary dysfunction in the human, anti-inflammatory agents may also have protective activity.

The therapeutic success of combining agents depends on determination of the right dosage combination and schedule. The nature of interactions between drugs is difficult to evaluate, and many different methods have been employed in such attempts (141). The combined action of two drugs can be categorized as simple addition, antagonism, or synergism. Too often such categorizations are made from data derived at only one or two dosage combinations, increasing the risk of erroneous or incomplete understanding of the interaction (141, 142). Reports by Takemura *et al.* (143, 144) illustrated the importance of evaluating an interaction over as wide a dosage range as possible. They showed that, depending on the dose, carbachol both inhibits and increases isoproterenol-induced amylase secretion in rat parotid gland. This lack of comprehensive dose-response information is a major deficiency of studies to date.

Clinical Strategies to Reduce Radiation-Induced Salivary Gland Damage

The success of drugs demonstrated by animal studies in reducing radiation damage to salivary glands has led to preliminary clinical trials in human patients. Amifostine (WR-2721), the free radical scavenger and cytoprotectant, has been tested in patients who received more than 45 Gy of radiation to treat head and neck cancer (145). The investigators administered the drug intravenously immediately prior to irradiation, and followed flow rates of unstimulated and stimulated whole saliva, as well as stimulated parotid saliva, over a period of up to 2 years. Salivary gland function improved over time after irradiation, but due to the limited nature of this study, amifostine's real effectiveness in protecting salivary function remains to be defined.

Pilocarpine has also been evaluated prospectively for its radioprotective effects in a small group of patients undergoing therapeutic head and neck irradiation (33, 146). Patients who received pilocarpine, 5 mg q.i.d. the day before and 3 months following irradiation, exhibited a lower frequency of subjective complaints and smaller reductions in salivary flow over the 12-month follow-up period compared to placebo-treated controls. Pilocarpine failed to protect a patient whose glands were irradiated completely, and some parotid gland tissue was shielded in three of the five patients of the pilocarpine group. For these reasons, the investigators attributed most of the difference to stimulation of salivary tissue outside the radiation field. Increased flow alone may not be a significant factor since natural variation in stimulated and unstimulated flow rate does not seem to affect radiation-induced salivary gland damage (147). However, a recent, retrospective study involving 40 patients in which both parotid glands were treated to a dose of at least 45 Gy suggests that pilocarpine may, indeed, provide some protection for irradiated tissue, at least as measured by subjective complaints associated with xerostomia (148).

Although the results of these studies are promising, the small number of subjects involved limits any conclusions about the effectiveness of these drugs in protecting the salivary glands from damage. It will also be essential to establish that drug pretreatment does not decrease the therapeutic effects of irradiation on the target malignant cells. More extensive clinical trials are necessary to establish the roll of protective drug pretreatment protocols in radiation therapy.

Both animal studies and clinical experience have established that two factors–radiation dose and volume of gland included in the radiation field–are the major determinants of the severity of radiation-induced salivary gland damage. The use of new computer-aided treatment planning to reduce these factors during radiation of the head and neck is producing encouraging results. Using computed tomography guidance to define precisely the target tumor and parotid gland volumes, three-dimensional (3D) or three-field conformal radiation therapy is designed to shape the spacial distribution of the radiation dose to maximize delivery to the target while minimizing irradiation of the parotid glands. A discussion of the details of this treatment modality is beyond the scope of this review, but initial results are encouraging and suggest that function of the major salivary glands can be protected without compromising therapeutic effects on the target tissues (149–152).

Finally, investigators at the National Institute of Dental and Craniofacial Research and Johns Hopkins University School of Medicine are using gene transfer techniques to re-engineer salivary ductal cells to secrete saliva by incorporating the gene for an aquaporin protein (153). These proteins form pores in cell membranes that allow fluid to pass through. Using an adenovirus to carry the gene, these investigators infected rats whose salivary flow rate was decreased 30% by irradiation. The amount of saliva secreted by rats infected with virus that contained the aquaporin gene was significantly greater than that of rats infected with virus that did not contain the gene (154). This technology is in its infancy and must be developed further before its clinical usefulness can be established.

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