Radioprotection of Oral Cavity Structures by S-2-(3-Aminopropylamino) Ethyl Phosphorothioate (WR-2721)

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RADIOPROTECTION OF ORAL CAVITY STRUCTURES
BY S-2-(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOATE (WR-2721)

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RADIOPROTECTION OF ORAL CAVITY STRUCTURES
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Studies reporting a high concentration of WR-2721 in mouse salivary glands led to our studies of possible radioprotection of these glands by this drug from ionizing radiation. Oral effects of radiation in the presence of WR-2721 were studied in mice and dogs. Histological evaluation of mouse salivary glands irradiated with 1000 rads of $^{60}$Co showed essentially no difference between control and experimental animals. Almost full regeneration of the serous salivary components occurred by 6 months in both groups and neither group had changes in the mucous glands. The use of higher doses of radiation in the mouse was prevented by the oral cavity death syndrome (LD50/8-10) which was reduced by a factor of 2.1 when WR-2721 was given 30 minutes before irradiation of the head. Salivary function in mongrel dogs measured at weekly intervals for one month following radiation showed no significant difference in control and experimental animals; therefore the salivary gland may be an organ capable of metabolizing or excreting of WR-2721. Marked protection from acute radiation damage of the skin and oral mucosa was observed in dogs receiving WR-2721 prior to treatment with radiation. A dose modifying factor of 1.67 was obtained for these structures. If such normal tissue sparing could be achieved clinically, higher doses of radiation could be used in treatment of head and neck malignancies, thereby increasing the probability for successful radiation therapy for such tumors.
INTRODUCTION

Cancer, characterized by potential invasion of healthy tissues, metastases to other parts of the body, and unlimited cell growth, is the second leading cause of death in the United States. In 1971, for example, 17.5 per cent of all deaths was due to cancer (4).

Cancer is also a major cause of death throughout the world. Survival rates for individuals with various types of cancer have been increased. The survival rate increase for individuals with some types of cancer has been striking, while in others, the survival rates have decreased or leveled off. The survival rates for individuals with certain forms of cancer have shown no change during the past twenty-five years. There has been an overall decrease in cancer incidence and mortality, but some types of cancer (e.g., lung, pancreas, colon, prostate, and bladder) have increased.

Recent significant progress in the area of cancer treatment can be explained by three factors: (1) diagnosis of the disease in an early localized state, (2) treatment of the cancer within four months of diagnosis, and (3) development of new diagnostic and therapeutic techniques and devices. It should be noted that, even with the new advances, many curable patients will continue to die of cancer because they escaped early detection and cannot benefit from modern therapy when their diseases are in early stages. As new diagnostic and therapeutic methods are developed and used, trends in treatment plans change. In the treatment of breast cancer, for example, survival rates recorded
during the period from 1950-1959 have been significantly improved (4).
Since the 1950's, patients have received less surgical treatment and
more adjuvant therapy with radiation, chemotherapy, and/or hormones.

This study is limited to the investigation of radiation effects
related to radiotherapy of head and neck cancers. Medical care is
concerned primarily with patient support during and after the stress
of treatment in patients with head and neck cancers. A careful medical
history and physical examination should be performed as an aid in provi­
ding the best medical care possible. During the course of patient
examination all bodily functions should be considered by the physician.
Pulmonary function and cardiac status, for example, should be carefully
evaluated and current and/or past medical or surgical diseases should
be noted. All these factors play an important role in deciding which
treatment plan will be followed. When it has been determined that
radiotherapy is applicable as a therapeutic means, a treatment plan
is made out which may require that the patient be treated with 6000–
7000 rads (radiation absorbed dose) of \(^{60}\)Co therapy delivered in frac­
tionated doses over a period of six to seven weeks.

Figure 1 shows, for example, a 52-year-old male patient who de­
developed an epidermoid carcinoma of the anterior tongue. Figure 2
shows the same patient six weeks later after he had received 6000 rads
of \(^{60}\)Co therapy through bilaterally opposed ports. The therapeutic
results were listed as satisfactory. Skin cancers detected early
usually require less total radiation for successful treatment. Figure
3 is a photograph of a patient who developed squamous carcinoma of the
skin. The treatment of this patient consisted of 5500 rads of \(^{60}\)Co
therapy delivered through a single lateral field over a period of five
Figure 1. 52-year-old male patient with an epidermoid carcinoma of the anterior tongue. Courtesy of Department of Radiation Medicine, University of Kentucky Medical Center.

Figure 2. Same 52-year-old male patient six weeks following eradication of the epidermoid carcinoma with 6000 rads of $^{60}$Co therapy. Courtesy of Department of Radiation Medicine, University of Kentucky Medical Center.
Figure 3. Male patient with a pre-auricular squamous carcinoma. Courtesy of Department of Radiation Medicine, University of Kentucky Medical Center.

Figure 4. Same male patient five weeks following eradication of the carcinoma with 5500 rads of $^{60}$Co therapy. Only a small pre-auricular ulcer remains. Courtesy of Department of Radiation Medicine, University of Kentucky Medical Center.
weeks. Figure 4 is a photograph of this patient taken after therapy for the cancer was completed.

An optimal selective effect in radiotherapy is reached when the tumor is completely destroyed without significant functional or structural injury to the surrounding normal tissue. It should be pointed out that a certain amount of damage to the normal tissue must usually be accepted along with the eradication of the malignancy, as a truly selective effect is not very often reached.

Since radiation spares no biological structure, the amount of radiation administered must be limited both in volume of tissue treated as well as in total dose during radiotherapy. The therapeutic ratio must be considered in order to keep the normal tissue injury at a minimum and still give an effective tumor dose. The therapeutic ratio is defined as the relationship between the tumor lethal dose and normal tissue tolerance (19). Radiotherapy is limited by this therapeutic ratio because no radiation effect is selective for the malignancy alone. Further studies dealing with the modification of the therapeutic ratio will therefore be likely to yield useful information. Modification of the therapeutic ratio might for example permit curing of non-curable malignancies because higher doses of radiation could be used. A useful modification of the therapeutic ratio could result from the use of agents which could protect the normal tissues while providing no protection to the cancerous tissues.

Chemicals that act as radiosensitizers and radioprotectors are known. Radiosensitizing agents enhance the radiation effects, whereas radioprotecting agents provide an apparent protection against the effects of radiation. These agents tend to have similar effects upon
normal and malignant tissues. An agent which could simultaneously protect the normal tissues and sensitize the malignant cells would be most useful, but no such agent is known. The present study is concerned with a drug, S-2-(3-aminopropylamino)ethyl phosphorothioate (WR-2721), which has been shown to protect normal tissues but not the malignant ones (17). This differential protection may be due, in part, to different concentrations of the drug in the healthy and malignant tissues. The purpose of this study is to evaluate the radioprotection afforded by this drug to head and neck structures in dogs and mice. Because high concentrations of this drug have been demonstrated in the salivary glands of mice, the present investigation evaluating the radioprotection afforded by this compound can indicate whether or not the compound has potential for use in radiation therapy of cancers.

Significant contributions to the knowledge of radiation effects have been made through the studies on compounds which modify ionizing radiation. Because of the severe toxicity which was produced in animal systems, early studies on radioprotectors were disappointing. The rebirth of interest in radioprotection in the United States can probably be accounted for by the discovery of a thiophosphate compound (WR-2721) which was found to provide protection to normal tissues but not to cancerous tissues in a tumor-bearing mouse following radiation (30). Thiophosphates have been the major compounds investigated for a role in radioprotection. The most prominent of these radioprotectors are listed in Table I (11).

The phosphorothioate radioprotective agents were first synthesized in 1959 (1). Later studies showed that these agents protect against ionizing radiation (2, 10, 28). Recent studies indicate that the new
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<td>MEA  β-mercaptoethylamine (cysteamine)</td>
<td>H₂NCH₂CH₂SH</td>
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<td>WR-2529  3-(2-mercaptoethylamino)propionamide p-toluenesulfonate</td>
<td>H₅SCH₂CH₂NHCH₂CH₂CNH₂·CH₂C₆H₄SO₂H</td>
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<td>WR-2721  S-2-(3-aminopropylamino)ethyl phosphorothioic acid hydrate</td>
<td>H₂NCH₂CH₂CH₂NHCH₂CH₂SPO₂H₂·XH₂O</td>
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<td>WR-2823  S-2-(5-aminopentylamino)ethyl phosphorothioic acid monohydrate</td>
<td>H₂N(CH₂)₅NHCH₂CH₂SPO₂H₂·H₂O</td>
</tr>
<tr>
<td>WR-638  Sodium hydrogen S-(2-aminoethyl)phosphorothioate</td>
<td>H₂NCH₂CH₂SP--(OH)(ONa)</td>
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radioprotective phosphorothioate compounds are less toxic than the previously studied radioprotectors. WR-2721 has been shown to give the greatest protection of any of these compounds (12, 17). Because WR-2721 has been shown to provide a high degree of protection in several normal tissues while providing only minimal protection for tumors, it has become a significant drug for use in studies of radio-protection. The toxicity produced by this drug is within an acceptable range.

The process by which WR-2721 enters the cell has been described (27). Upon entry into the cell, the compound becomes dephosphorylated enzymatically, thus exposing the sulfhydryl group. It is thought that this process occurs fast enough that no intact WR-2721 is left inside the cell. Normal cells and tumor cells show identical cell membrane permeability to this drug, and both cell types readily dephosphorylate the compound. However, since the drug enters the cell by passive diffusion, less absorption would be expected to occur in a solid tumor because the tumor is poorly vascularized. This prevents delivery of large concentrations of WR-2721 to the tumor cells (12).

The mechanism by which WR-2721 protects the cell from radiation is not certain. It is thought that it may work by the same mechanism(s) proposed for all sulfhydryl radioprotectors (7): (1) removal of free radicals formed by radiation, (2) repair by donation of hydrogen atoms, (3) interaction with cellular proteins, (4) production of hypoxia in the tissues, and/or (5) delay of cellular division or inhibition of mitosis.

If the mechanism is by free radical removal, then it is suggested that free radicals produced by radiation, such as the hydroxyl radical,
oxidize the protective agent, therefore forming a resonance-stable free radical which is not capable of reacting with the cellular components. If the free radicals act as oxidizing agents they are therefore prevented from reacting with cellular components. It was found that the oxidation of radioprotectors, including WR-2721, indicates that they are capable of reacting with the radicals which are produced by the radiation (15).

It is possible that the protective agent can donate a hydrogen atom to a molecule which was converted to a free radical by radiation. Thus the injured molecule would be restored to its original or normal state upon acceptance of the hydrogen atom (6).

If the protective agent reacts with cellular proteins, the free sulfhydryl groups could form mixed disulfide bonds with the sulfhydryl groups of the proteins. Upon attack by a radiation-induced free radical, one sulfur atom of the disulfide is oxidized while the other is reduced. Consequently, the protein is not damaged by radiation. This mode of action is illustrated in Table II. It should be noted that the protein could be oxidized which would leave the protein altered. This alternative route would allow for only 50 per cent of the radical interactions to damage proteins, hence leaving 50 per cent of the proteins protected from this type of oxidation (7).

The damaging effects of radiation are enhanced in the presence of oxygen, and a state of hypoxia provides protection from the radiation. Since thiol compounds are very easily oxidized, it has been theorized that tissue oxygen would be used up during such oxidations, hence leaving a state of hypoxia. One investigation tended to support this theory (29). This study showed that upon injection of WR-2721
TABLE II

POSSIBLE RADIOPROTECTIVE MECHANISM WITH MIXED DISULFIDE FORMATION

ALTERNATIVE ROUTE

Oxidation of proteins takes place when this alternative route occurs.
Vasodilation is produced and is prominent in the spleen. The vasodilation of the spleen alters the blood supply to the rest of the body and does so in such a way as to reduce tissue oxygen tensions and thereby elevates radiation resistance. It was pointed out that vasodilation may be a universal effect of WR-2721, but if this is so, it should not prevent the use of WR-2721 in radiotherapy in man. In the event vasodilation does not take place, a larger amount of WR-2721 would be expected to be tolerated.

If the mechanism involves a delay in cell division, there may be more time available for repair of damage caused by radiation. If the mechanism involves mitotic inhibition, it could be in such a way as to arrest the cells in a radioresistant stage, therefore providing protection from the radiation.

Because there are exceptions to all the above mechanisms, the radioprotection provided by WR-2721 probably results from a combination of several mechanisms. There are, for example, compounds which form disulfides but give no protection (7). Cysteamine has shown protection unrelated to hypoxia (7). There are molecules with functional group distributions in structures similar to those in the resonance-stable free radicals formed by radioprotectors which do not give protection (7). More research is needed before the mechanism or mechanisms through which protection is afforded can be explained adequately.

Metabolism studies in rats (22) have indicated that the main excretion of WR-2721 occurs through the feces. The second most important route for excretion is in the urine. About 74 per cent of the total drug administered was excreted during the first 24 hours following oral administration of WR-2721 and about 94 per cent after 48 hours. Only
about 6 per cent was retained. It was found that about 1.35 per cent of the total drug retained was concentrated in the selected organs (e.g., eyes, brain, heart, lung, liver, kidney, spleen), 0.09 per cent by the blood, 0.01 per cent by the muscle, 0.02 per cent by the fat, 1.29 per cent by the gastrointestinal tract, and 3.31 per cent by the carcass and remaining viscera. Observations of the excretory products during the 48 hours following administration showed both the urine and feces to be of normal color and consistency. Autopsy of the animals after 48 hours showed all organs to be normal except the lungs of one animal (a total of three was used) which appeared splotchy in color.

A second study (23) was done using twice the amount of WR-2721 per gram of body weight with essentially the same results as those listed above.

The studies mentioned above reported that the drug crosses the blood-brain barrier, but that the amount crossing the barrier was less than 0.01 per cent of the total amount administered to the animal. This figure included the combined amounts which enter the eyes and brain. In a recent study, it was reported that the brain, one hour following intravenous injection of WR-2721 shows very little concentration of the drug (25). An earlier study reports that the drug WR-2721 does not cross over the blood-brain or placental barriers (20). However, radioactivity was reported around the cerebral blood vessels and choroid plexus. Nevertheless, this radioactivity seemed to be very low.

High localization of WR-2721 has been reported in the hematopoietic tissues, gastrointestinal and urinary tracts, and in some glandular tissue with maximum concentrations in the tissues between 1-4 hours following administration (20). The blood appears to be cleared of the
drug within 5 minutes. The major site of metabolism of the drug seems to be the liver with most of the elimination of the drug taking place via the gastrointestinal and urinary tracts within 48 hours following administration.

The main route of excretion of WR-2721 seems to be via the gastrointestinal and urinary tracts. The liver shows high concentrations of the drug, and it appears to be the area of greatest metabolism of the drug. Other sites of high concentrations include the hematopoietic tissues and glandular tissues. The submandibular salivary gland has been shown to have a great density of the drug (25).

Autonomic and acute cardiovascular effects of WR-2721 have been found to be slight with cumulative intravenous doses of up to 600mg/kg. These effects were studied in cats and dogs (5). Only small increases in femoral blood flow were produced by WR-2721 whereas a similar phosphorothioate produced a marked increase. A slight depression in blood pressure of 5-15 mm Hg was noted which lasted for 5 minutes but was followed by a gradual increase of 30-40 mm Hg. A depression of 15-30 heart beats per minute was obtained from WR-2721. The carotid artery occlusion pressor response was reduced by WR-2721 thirty minutes following injection, whereas it was not reduced by the other similar phosphorothioates. Lower doses (one-half as much) of the other phosphorothioates produced much greater depressive effects which lasted for longer periods of time. This indicates that WR-2721 is not as toxic as similar phosphorothioates.

Indications that WR-2721 may act as a ganglionic blocking agent are evidenced by its ability to differentially block the effects of pre-ganglionic nerves leaving the post-ganglionic nerves unstimulated.
Other effects noted from WR-2721 injection included slight mydriasis and a watery type salivation.

During the course of radiotherapy of head and neck cancers, complications may be introduced which are associated with the skin, mouth and throat, and salivary glands (15). With the use of high energy sources such as $^{60}$Co gamma rays, skin reactions are usually minimal, whereas with kilovoltage x-rays (orthovoltage) intense skin reactions may be noted. Two to four weeks following radiation is the period of most intense skin reactions. At low radiation doses, erythema, pigment loss, and dry desquamation may develop. At higher doses, moist desquamation and late necrosis may develop. The area affected in skin reactions usually conforms with the field size used during the treatment.

Dryness of the mouth and throat is a common complaint of patients receiving head and neck radiation. Difficulty in swallowing may accompany the dryness. Thrush may develop within the mouth following irradiation with $^{60}$Co or x-ray. The dryness is due to the radiation effects on the salivary glands.

Salivary glands are classified as highly radiosensitive organs because of the rapid effect radiation has on their function. But, since epithelial glands divide only rarely, the salivary glands could be expected to be radioresistant (18).

As early as one day post-irradiation, the salivary glands may show rapid and marked swelling. This effect may be accounted for by the changes in the endothelium of the vasculature, congestion and increased permeability of capillaries, interstitial edema and acute inflammatory cell infiltration, narrowing of the excretory duct due to edema and inflammatory pressure, swelling of duct linings or plugging of
lumina by mucous. These changes could stop or alter the function of the salivary glands. Necrosis and cell degeneration may be a consequence.

Human salivary glands surgically removed 24 hours following single doses of radiation show a parallel between the location and degree of acute inflammatory cell infiltration and cell degeneration. These two changes occur mostly in the serous cells and are considered dose-dependent. Inflammatory infiltration includes invasion by polymorphonuclear leukocytes, eosinophilic leukocytes, and plasma cells along the septa and around acini (alveoli). Sometimes lumina appear to be full of exudate from degenerating leukocytes and acinar cells. Degenerative vascular changes appear in the cells, producing vagueness in acinar outline and cleavage of the cells from their basement membranes. Pyknosis is present. Zymogen granules pool in acini and some glands show a decrease in zymogen granules.

The primary damage seems to occur in the serous cells, although the mechanism of tissue reaction is not known. It may be that serous secretion (with the enzyme amylase) could enter capillaries altered by radiation causing the inflammatory response noted earlier since there are capillaries between serous acinar cells and not in mucous cells. Hence the serous epithelial degeneration could be a secondary effect of radiation. The lack of mucous cell degeneration could be due to the fact that the mucous secretion does not enter the altered capillaries if indeed this is the case.

The changes mentioned above for single doses also appear if the radiation is delivered in fractionated doses, but the changes develop progressively.
Acinar cells show transient or abortive signs of secretion or mitotic regeneration, but these are followed by progressive degenerative processes. Recovery of ducts is usually more marked. The cells lost earlier by the excretory ducts, for example, are replaced by mitotic activity. The excretory duct epithelium later show squamous metaplasia or hyperplasia. Functional inefficient acini may be produced by the intercalated ducts as acini are progressively lost.

The salivary glands are firm, smaller than normal, and strongly adherent to the surrounding tissues at the end of the acute clinical period. The main histological changes at the end of this period include: (1) interlobular fibrosis, (2) interstitial fibrosis and sclerosis with infiltration of inflammatory cells, (3) loss and atrophy or involution of acini, (4) foci of necrosis in acini, and (5) metaplasia.

There are three major pairs of salivary glands in all mammals (19). These compound tubulo-alveolar glands are known as (1) parotid, (2) sublingual, and (3) submandibular. The salivary glands are composed of serous and mucous cells. The parotid glands are composed entirely of the serous type. Although the submandibular and sublingual glands are both mixed glands (serous and mucous), the submandibular glands are almost all serous, whereas the sublingual glands are almost entirely mucous. The typical gland consists of a secretory portion, the alveolus, connected to a system of tubes which terminate with an opening in the oral cavity (3). Connective tissue septae run through the gland separating groups of alveoli into gland lobules. Narrow channels, the intercalated ducts which are composed of cuboidal cells, lead
directly from the alveoli.

While these ducts are long in the parotid glands, they are short in the submandibular and sublingual glands and even sometimes absent in the sublingual glands. Numerous infoldings of the basal part of the cell membrane, associated with mitochondria, forms the striated ducts which lead from the intercalated ducts. Striated ducts lead to the larger interlobular ducts which lead to the intralobular ducts which in turn unite to form a single, terminating excretory duct composed of columnar epithelium. Toward the opening of the excretory duct in the oral cavity, the epithelium becomes pseudostratified.

The excretory duct of the parotid gland opens into the vestibule of the mouth just opposite the second molar tooth. The excretory duct of the submandibular gland opens near the side of the frenulum of the tongue. Parts of the sublingual gland open into the excretory duct of the submandibular while other parts open through a single excretory duct near the opening of the submandibular gland.

The secretory cells of the glands are both mucous and serous. Zymogen granules, intracellular concentrations of ptyalin, give the cytoplasm of serous cells a granular appearance. The rounded nucleus is found toward the basal end of the cell. The basal end of the cell is strongly basophilic due to a high content of RNA. Associated with this area are the ribosomes of the endoplasmic reticulum which produces the protein of the zymogen granules (24).

The cytoplasm of the mucous cell stains very lightly when stained with hematoxylin and eosin. The mucinogen (precursor of mucin) contained within the mucous cell gives the cell its characteristic "empty" look upon staining. Usually this mucinogen is lost during preparation
for staining, hence the cytoplasm has a vacuolated appearance. The nucleus of the mucous cell is flattened and close to the base of the cell.

A good blood supply furnishes the salivary glands their nourishment. The larger vessels are found in the septae between the lobes and a capillary plexus forms around the alveoli. Both sympathetic and parasympathetic nerves innervate the salivary glands.

The mucous cells secrete mucin which is a clear, viscous substance. Secretion of serous cells is thin and watery consisting of an electrolyte solution. The parotid gland secretes ptyalin, an amylase responsible for splitting starch and glycogen enzymatically into maltose. Upon stimulation of either the sympathetic and parasympathetic nerves, a flow of "juices" is produced. Nerve stimulation also increases the flow of blood to the glands (21). Saliva contains electrolytes with the main ones being Na\(^+\), K\(^+\), HCO\(_3^-\), and Cl\(^-\). The composition of saliva is usually related to the nature of the food eaten.

Approximately one liter of saliva is produced daily by man. The submandibular glands produce two-thirds of this amount, the parotid glands produce one-fourth with the remainder produced by the sublingual and other small glands (buccal). Saliva may be modified by the striated ducts with their secretion of water and inorganic salts. Saliva is important for aid in swallowing and tasting food. It also helps maintain oral hygiene. Salivary glands play an enzymatic role; for example, ptyalin, mentioned earlier. An antimicrobial role is also attributed to saliva, probably because of the thiocyanate ion present.

The salivary glands have been shown to have a high concentration of WR-2721 following administration of the compound. The salivary glands
are nearly always involved during the radiotherapy of head and neck tumors. WR-2721 has been shown to afford a high radioprotective effect to some normal tissues while providing only minimal protection to the tumor; this seems to be related to different concentrations of the drug in the two different tissues. This study is concerned with the evaluation of this drug for the possible radioprotection afforded to the salivary glands, skin, and oral mucosa of the oral cavity.
MATERIALS AND METHODS

Mice were used to study the oral radiation death syndrome and the effects of radiation on the histology of the salivary glands with and without WR-2721. All mice used were female, white, and of inbred strain Balb/c. They were purchased at the age of 5-7 weeks from the Texas Inbred Company, Houston, Texas. The mice were not used for experiments until they were at least 7-9 weeks old. All mice were housed in 29 cm long x 18 cm wide x 12.5 cm high polystyrene cages. They were fed and watered ad libitum. The average weight of the mouse was 20 grams.

The mice were divided into two major groups for the oral radiation death study. One group received WR-2721 (supplied by Dr. Melvin Heiffer, Walter Reed Army Hospital, Washington, D.C.) and radiation while the other group received only radiation. The drug-treated (experimental) group was anesthetized with 0.05 mg Nembutal Sodium Solution, U.S.P. (sodium pentobarbital, 50 mg/ml) injected intraperitoneally (Jelco sterile, disposable syringes with Jelco sterile, disposable 25 gauge, 5/8" bevel needles) 45 minutes prior to radiation. Fifteen minutes later (30 minutes prior to radiation) this group was injected with 500 mg/kg WR-2721 intraperitoneally. The drug was dissolved in Bacteriostatic Sodium Chloride Injection, U.S.P. (Ambot Solutions) and filter sterilized by suction filtration through a 0.20 micron plain membrane Nalge filter unit just prior to injection. The non-drug-treated (control) group was injected with 1.0 mg Nembutal intraperitoneally 30 minutes prior to radiation treatment.
Both control and experimental groups received $^{60}\text{Co}$ radiation, to the head only, using a 28 cm x 28 cm field blocked off with lead (0.20 cm thick) to produce a 4 cm x 20 cm field and a source-to-skin distance (SSD) of 65 cm. The mice were irradiated at a rate of approximately 295 rads/min with a Zoneguard Picker V-9 $^{60}\text{Co}$ model with a f factor (roentgens/rads) of 0.957, displacement factor of 0.985, chamber correction factor of 1.026, and a stem correction factor of 1.0009 (when calibrated at 8 cm x 8 cm).

The experimental mice were divided into groups of ten each and were irradiated with 100 rad increments for each successive group from 3000-3700 rads. The control mice were also divided into groups of ten each and were irradiated with 100 rad increments for each successive group but ranging from 1200-1800 rads.

Following radiation the mice were observed for deaths for a period of two weeks. Those deaths occurring from day 0-2 following radiation treatment were related to toxicity and these animals were not used in the study. The per cent dead was plotted against radiation dose and the method of Litchfield and Wilcoxon (13) was applied to determine the LD50/8-10.

Mice used for examination of histological radiation effects on salivary glands were divided into two groups. One group received 500 mg/kg WR-2721 and radiation while the other group received only radiation. The mice were irradiated in the same manner as previously described for the oral radiation death study.

Mice were sacrificed with an overdose of ether at different time intervals following radiation: 1 day, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, and 6 months. Salivary glands
were excised and placed immediately in Bouin's fixative. After being in the Bouin's fixative for 4 hours, the glands were transferred into 70 per cent ethyl alcohol for an indefinite period of time in excess of 15 minutes. Dehydration was completed with three changes of 95 per cent ethyl alcohol for 15 minutes each followed by three changes in 100 per cent ethyl alcohol for 15 minutes each.

The tissue blocks were then cleared in two changes of chloroform for 15 minutes each. After clearing, the glands were infiltrated with two changes of Paraplast paraffin (melting point of 56-57°C) for 15 minutes each in a Preiser Thelco Model #17 heating oven (57°C). This was followed by infiltration in another change of paraffin for 15 minutes in a Labline Vacuum Oven (16-24 inches water vacuum).

The hardened blocks of tissue were sectioned both longitudinally and transversely into 5 micron sections with an AO Spencer "820" rotary microtome. The sections were allowed to fall into a water floatation bath maintained at 44-45°C. The sections were then put onto albuminized (Mayer's Albumin Fixative) glass slides. These glass slides were then placed into a heating oven at 57°C and allowed to stay until the paraffin melted.

The slides were then placed into three changes of xylene for 2 minutes each and then the tissue sections were rehydrated with absolute ethyl alcohol, 95 per cent ethyl alcohol, and finally water. Rehydration steps consisted of three changes of each of the above solutions for 2 minutes each.

After rehydration, different sections from the same tissue block were stained using three different stains: (1) hematoxylin and eosin, (2) Masson-trichrome, using aniline blue as the counterstain, and
(3) mucicarmine, using freshly made mucicarmine prepared by the modified Mayer's method (8); the regular Mayer's method was then used (14).

After staining was completed, glass cover slips were applied with Harleco Synthetic Resin Mounting Medium. The sectioned salivary gland preparations were then examined with light microscopy.

Dogs were used to study the effects of radiation on salivary function, skin, and oral mucosa with and without WR-2721. Both female and male beagel dogs were used. They were maintained on ad libitum feeding in 129 cm long x 97 cm wide x 74 cm high aluminum-barred pens. The average weight of the dogs used was approximately 18 kilograms.

Eight dogs were divided into four groups of two dogs each. One dog from each group received radiation and WR-2721 while the other dog received only radiation. Both non-drug-treated (control) and drug-treated (experimental) dogs were anesthesized with 26 mg/kg veterinary grade Nembutal (60 mg/ml) injected intravenously (20 gauge, 1 1/2" bevel needles) prior to radiation. The experimental dogs were injected intravenously with 70 mg/kg WR-2721 30 minutes prior to radiation. The drug was dissolved just before use and filter sterilized as described above.

Both control and experimental dogs received radiation, to the left side of the head only, using a 16.5 cm x 20 cm field blocked with lead (0.6 cm thick) to a field of 8.25 cm x 10 cm, thereby excluding radiation to the right side of the dog. During the radiation treatment the dog was placed in a supine position with the bottom of the lower jaw facing the x-ray radiation tube. The dogs were irradiated with a SSD of 50 cm at a rate of approximately 122 rads/min using a 20 milliamp,
280 kilovolt power Picker X-Ray (Orthovoltage) machine with a f factor of 0.948, half-value layer of 1.32 mm copper, and an effective energy of 88.5 kilo-electron volts. Lithium fluoride chips were placed inside the cheeks of both the right and left sides of the dogs and the doses of radiation were verified with a Victoreen 2800 Thermoluminescent Dosimeter Reader. Each of the four groups of dogs received a single dose of radiation. The doses ranged from 1000 to 2500 rads at 500 rad increments.

Post-irradiation salivary gland secretions were measured at weekly intervals for one month following radiation. Measurements were taken for both the right (non-irradiated) and left (irradiated) sides of both the control and experimental groups.

The measurements were taken with the dogs in supine positions under Nembutal anesthesia. The mouths of the dogs were first dried with gauzes, then pre-weighed Curity gauzes were placed in the buccal pouches of both the right and left sides. Gauzes were also placed sublingually. The mouth was tied loosely with a gauze. Prostigmin 1:2000 (0.5 mg/ml) was injected intravenously at a dose of 1.0 mg per dog. The gauzes were removed 15 minutes later and reweighed. The difference in the gauze weight before and after salivary collection was used to represent salivary flow from the right and left buccal pouches. Sublingual gauzes were discarded. These collected flows were plotted as a function of the time following radiation. Photographs were taken with a Nikkormat FTn camera, using Kodak Ektachrome-X ASA 64 color slide film, to record the acute reactions of the hair, skin, and oral mucosa.
RESULTS

The LD50/8-10 for the control group of mice was calculated to be 1600 rads (Figure 5), whereas the LD50/8-10 for the experimental group of mice was found to be 3354 rads (Figure 6). The dose modifying factor (DMF) is 2.1, calculated as the ratio of the LD50 experimental to the LD50 control group.

The toxic effect of combined Nembutal and WR-2721 was great in the mice. It was found that the amount of Nembutal required for anesthesia in the experimental group was one-half the amount required to anesthetize the control group.

The radiation dose of 2000 rads resulted in early death of all control mice and histological comparison at this radiation dose could not be made. Comparison of salivary glands irradiated with 1000 rads in the control and experimental mice shows similar histological changes in the serous glands (Table III). Mild degeneration appears as early as day one, progressing to early regeneration of the serous portion beginning at week two. Full regeneration of the serous portion appears to not be completed at six months following treatment. Patchy areas of normal serous cells and other areas of severe disorganization are seen in both groups.

The mucous glands in the control group show mild radiation damage after one month and full recovery appears at six months. In the experimental group, 1000 rads fails to produce any apparent damage to the mucous cells through the six month period.
Figure 5. Oral radiation death without radioprotection showing the LD50/8-10.
ORAL RADIATION DEATH (Without WR-2721)

DOSE (rads)

PERCENT DEAD
Figure 6. Oral radiation death with radioprotection showing the LD50/8-10.
ORAL RADIATION DEATH (With WR-2721)

DOSE (rads) vs. PERCENT DEAD
**TABLE III**

**RADIATION HISTOLOGY OF SEROUS GLANDS AFTER RECEIVING 1000 RADS**

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Without WR-2721</th>
<th>With WR-2721</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ONE DAY</strong></td>
<td>Not Remarkable</td>
<td>Not Remarkable</td>
</tr>
<tr>
<td><strong>ONE WEEK</strong></td>
<td>Some Degenerative Changes; Some Lack of Organization</td>
<td>Some Degenerative Changes; Some Nuclear Hyperchromatism</td>
</tr>
<tr>
<td><strong>TWO WEEKS</strong></td>
<td>Cellular Hyperplasia; Some Degenerative Changes; Hyperchromatic Nuclei</td>
<td>Cellular Hyperplasia</td>
</tr>
<tr>
<td><strong>THREE WEEKS</strong></td>
<td>Not Remarkable</td>
<td>Some Degenerative Changes</td>
</tr>
<tr>
<td><strong>ONE MONTH</strong></td>
<td>Not Remarkable</td>
<td>Not Remarkable</td>
</tr>
<tr>
<td><strong>TWO MONTHS</strong></td>
<td>Cellular Hyperplasia; Degenerative Changes; Nuclear Hyperchromatism</td>
<td>Cellular Hyperplasia; Nuclear Hyperchromatism</td>
</tr>
<tr>
<td><strong>THREE MONTHS, EIGHT DAYS</strong></td>
<td>Hydropic Degeneration; Hyperchromatic Nuclei</td>
<td>Cellular Degeneration; Hyperchromatic Nuclei</td>
</tr>
<tr>
<td><strong>FOUR MONTHS, ELEVEN DAYS</strong></td>
<td>Hydropic Degeneration; Hyperchromatic Nuclei</td>
<td>Cellular Degeneration; Cellular Hyperplasia; Nuclear Hyperchromatism</td>
</tr>
<tr>
<td><strong>FIVE MONTHS</strong></td>
<td>Hydropic Degeneration; Large, Hyperchromatic Nuclei</td>
<td>Some Hydropic Degeneration</td>
</tr>
<tr>
<td><strong>SIX MONTHS</strong></td>
<td>Cellular Hyperplasia; Nuclear Hyperchromatism</td>
<td>Cellular Hyperplasia; Nuclear Hyperchromatism</td>
</tr>
</tbody>
</table>
Preliminary baseline salivary flows were inconsistent in all dogs. The post-radiation saliva measurements of both control and experimental dogs do not show any consistent flow patterns (Figures 7 through 10). There is a general trend however of lesser salivary secretion from the left side of both control and experimental dogs.

There was a loss of pigment in the left buccal pouches of both control and experimental dogs with the maximum loss occurring at one month following radiation. There was almost total loss of pigment in the control dog (Figure 11) followed by full restoration of pigment within two months following radiation (Figure 12). Loss of pigment in the experimental dog was very slight one month following radiation (Figure 13). This was also followed by full restoration of pigment within two months following radiation (Figure 14).

The oral cavity structures at no time showed radiation damage following 1000 rads. Very minimal hair loss occurred at this low dose in the control animal, whereas there was no hair loss in the experimental dog.

Moderate hair loss occurred at 1500 rads in the control dog at two months, whereas there was no loss of hair in the dog receiving the drug. There was no hair loss in the experimental dog two months following treatment with 2000 rads (Figure 15), whereas the control member of the pair had total hair loss at that time (Figure 16).

Moist desquamation of the skin occurred at the 2500 rad level in the control animal. Figure 17 shows the control dog two weeks following 2500 rads. Figure 18 shows the same dog with moist desquamation one month following 2500 rads. Figure 19 shows the experimental dog two weeks following 2500 rads. Figure 20 shows the same dog with only
Figure 7. Salivary secretion measurements by weight following 1000 rads of x-ray (orthovoltage) radiation.

Legend

----- Without WR-2721
——— With WR-2721
  ○ Right Side
  △ Left Side
Figure 8. Salivary secretion measurements by weight following 1500 rads of x-ray (orthovoltage) radiation.

Legend

----- Without WR-2721
----- With WR-2721
 o Right Side
 Δ Left Side
Figure 9. Salivary secretion measurements by weight following 2000 rads of x-ray (orthovoltage) radiation.

Legend

----- Without WR-2721
——— With WR-2721
o Right Side
Δ Left Side
Figure 10. Salivary secretion measurements by weight following 2500 rads of x-ray (orthovoltage) radiation.

Legend

----- Without WR-2721
      With WR-2721
  o  Right Side
  △ Left Side
Figure 11. Left buccal pouch showing example of pigment loss in control dogs one month following x-ray (orthovoltage) radiation.

Figure 12. Left buccal pouch showing example of restoration of pigment in control dogs two months following x-ray (orthovoltage) radiation.
Figure 13. Left buccal pouch showing example of little pigment loss in the experimental dogs one month following x-ray (orthovoltage) radiation.

Figure 14. Left buccal pouch showing example of restoration of pigment in the experimental dogs two months following x-ray (orthovoltage) radiation.
Figure 15. Experimental dog two months following 2000 rads of x-ray (orthovoltage) radiation showing no hair loss.

Figure 16. Control dog two months following 2000 rads of x-ray (orthovoltage) radiation showing total hair loss of the left (irradiated) side.
Figure 17. Control dog two weeks following 2500 rads of x-ray (orthovoltage) radiation.

Figure 18. Control dog one month following 2500 rads of x-ray (orthovoltage) radiation showing moist desquamation of the skin.
Figure 19. Experimental dog two weeks following 2500 rads of x-ray (orthovoltage) radiation.

Figure 20. Experimental dog one month following 2500 rads of x-ray (orthovoltage) showing moderate hair loss on the left (irradiated) side.
moderate hair loss one month following 2500 rads.

The oral mucosal reactions were consistently greater in the control animals. The maximum acute reactions occurred at two weeks following radiation. Animals receiving WR-2721 had faster healing of the effects of radiation.

Figures 21 through 24 show the oral mucosal reactions of the control dog following treatment with 1500 rads. The experimental dog showed only a very slight reaction, similar to that seen in Figure 23, which appeared at two weeks following radiation and was gone by three weeks. The reaction in the control dog was still present at three weeks, but it was gone by four weeks following radiation.

The oral mucosa of the experimental dog receiving 2000 rads showed a larger reaction, than the dog receiving 1500 rads alone, at two weeks following radiation (Figure 25). All the oral mucosal radiation reaction in the experimental dog receiving 2000 rads was gone by one month following radiation (Figure 26). The control dog showed a much larger radiation reaction, covering the entire area of the oral mucosa irradiated, two weeks following radiation (Figure 27). A slight reaction was still present at one month following radiation (Figure 28).

Following 2500 rads the control and experimental dogs showed similar reactions of the oral mucosa with the reaction covering the total area irradiated. But the reaction occurred at one week in the control dog and did not occur until two weeks in the experimental dog. Figure 29 shows the reaction in the control dog one week following radiation. Figure 30 shows the reactions still present at four weeks following radiation. Figure 31 shows the reaction in the experimental dog at two weeks following radiation. Figure 32 shows the reactions
gone by three weeks following radiation. A similar oral mucosal reaction effect occurred at 2500 rads in the drug-treated animal and at 1500 rads in the non-drug-treated animal. The ratio of these two radiation doses gives a DMF of 1.67.
Figure 21. Oral mucosa of the tongue in the control dog one week following 1500 rads of x-ray (orthovoltage) radiation.

Figure 22. Oral mucosa of the tongue in the control dog two weeks following 1500 rads of x-ray (orthovoltage) radiation.
Figure 23. Oral mucosa of the tongue in the control dog three weeks following 1500 rads of x-ray (orthovoltage) radiation showing only a small area of radiation reaction.

Figure 24. Oral mucosa of the tongue in the control dog one month following 1500 rads of x-ray (orthovoltage) radiation. Mucosa is free of any radiation reaction.
Figure 25. Oral mucosa of the tongue in the experimental dog two weeks following 2000 rads of x-ray (orthovoltage) radiation.

Figure 26. Oral mucosa of the tongue in the experimental dog one month following 2000 rads of x-ray (orthovoltage) radiation.
Figure 27. Oral mucosa of the tongue in the control dog two weeks following 2000 rads of x-ray (orthovoltage) radiation.

Figure 28. Oral mucosa of the tongue in the control dog one month following 2000 rads of x-ray (orthovoltage) radiation.
Figure 29. Oral mucosa of the tongue of the control dog one week following 2500 rads of x-ray (orthovoltage) radiation.

Figure 30. Oral mucosa of the tongue of the control dog four weeks following 2500 rads of x-ray (orthovoltage) radiation.
Figure 31. Oral mucosa of the tongue of the experimental dog two weeks following 2500 rads of x-ray (orthovoltage) radiation.

Figure 32. Oral mucosa of the tongue of the experimental dog three weeks following 2500 rads of x-ray (orthovoltage) radiation.
DISCUSSION

Even though the reason for the oral radiation death syndrome is not known, a DMF of 2.1 could be of great significance. This syndrome, which has been described only in mice (9), has been modified to a larger degree by WR-2721 than any protective compound previously studied. This death point (oral radiation death) may have little significance in large animals, but it prevented the study of the effects of large doses of radiation on the histology of mouse salivary glands, as originally planned. The desire to use mice for the study of the effects of radiation on salivary gland histology stems from the fact that use of these small inbred animals would allow comparison studies of large numbers of genetically identical animals. Histological evaluation of salivary glands in this study is therefore limited to low radiation dose in mice for acute and later changes. Higher doses can be evaluated in mongrel dogs.

The salivary gland may be an organ for excretion or metabolism of WR-2721 which could explain the lack of protection observed in the salivary serous glands. It is apparent that the mucous glands are relatively radioresistant because only mild radiation damage, followed by full regeneration, was observed in the control mice. This is also observed clinically in patients receiving radiation to the salivary glands. There is a quick reduction in the serous secretions, leaving the patient with thick mucous secretions (26).
Physiological changes in both the control and experimental dog salivary glands were smaller than expected. The radiation doses given far exceeded the equivalent doses used in human radiation therapy. Salivary secretion from the irradiated glands in both experimental and control animals however continued, although at decreased levels.

Possible problems with this study might include:

1. There is unusual radioresistance of dog salivary tissue as compared to human salivary tissue.
2. The method of saliva collection was inadequate.
3. Less than the intended dose of radiation was delivered.
4. Diurnal variation masked the results.
5. The high concentration of $^{35}$S WR-2721 seen in the autoradiographs of the salivary glands represents excretion of this drug, similar to excretion observed in the kidney (25).

It is most likely that the salivary gland is another organ of excretion for WR-2721. If this is true, then possibly no protection would be expected. The kidney also shows high concentrations of $^{35}$S WR-2721, but it is not protected by the drug. The kidney merely excretes the drug.

It is felt that the method of saliva measurements was adequate. The mouth was dried with gauze before insertion of pre-weighed gauzes. Gauzes were also placed sublingually to prevent the saliva secreted there from draining onto the pre-weighed gauzes in the buccal pouches. Since the gauzes used in the pouches did not become supersaturated and the mouth was quite dry after they were removed, it seems the full amount of saliva secreted was obtained.

Both lithium fluoride dosimetry and the radiation changes observed
in the skin and oral cavity indicate the actual doses delivered were the planned radiation doses.

Preliminary salivary secretions were collected both in early morning and late evening, with the dogs on ad libitum feeding, and twelve hours without food. No pattern could be found after these manipulations. The mongrel dogs of course could not be compared with each other in the same manner as inbred mice were compared.

Protection of the skin and oral mucosa was readily apparent at two to four weeks in dogs receiving doses higher than 1000 rads. The end point for comparison between the control and the experimental animals that was chosen was that effect which would be clinically acceptable. Although there was protection against moist desquamation at the 2500 rad level, this condition is rarely observed in clinical radiotherapy unless bolus material for electron build-up is placed directly on the skin.

The total-hair-loss end point resulted in a DMF of 1.67. Higher DMF's of 2.0 and 2.4 have been reported for skin protection (17, 30), therefore problems with this study might include:

1. The period of thirty minutes between intravenous injection of the drug and radiation was too long. This time period was chosen to allow greater concentration of the drug in the salivary glands. Autoradiographic studies of $^{35}$S WR-2721 shows that the skin clearing of the drug is rapid (25), therefore, higher DMF's for skin and oral mucosal protection might have been obtained if the radiation had been delivered 5-10 minutes following the drug injection.

2. Large field sizes in large animals have not been studied
previously for skin protection. It is possible that the protective effect might be greater when smaller areas are irradiated.

3. The orthovoltage machine used in this study would be expected to produce a higher reaction at the skin surface than megavoltage machines.

It seems that WR-2721 has a definite protective action against ionizing radiation in oral cavity structures. The protection is greater at lower reaction grades than at the high-dose end point of moist desquamation. It is most probable that the protective effect could be greater if the time between the drug injection and radiation were less.

One of the most common complaints of patients receiving head and neck radiation is a dry mouth due to the radiation damage to the salivary glands. Although this drug in this study was not shown to provide protection of the salivary serous glands, it does give promise of being useful clinically. The patient could possibly be protected from the acute radiation reactions of the skin and oral mucosa using WR-2721.

The toxicity noticed in the mice receiving Nembutal and WR-2721 was not apparent in the dogs. This probably is related to the size of the animal and the amount of WR-2721 given. This would not be a problem with humans, as the patient is rarely anesthetized prior to radiotherapy.

Additional investigations of the radioprotective effect of WR-2721 are indicated on the basis of findings reported in this study. Some possible suggestions for new studies might include:

1. The protective effect may be related to the field size of radiation. Studies with different field sizes could therefore prove to be valuable.
2. The protective effect may be different when different radiotherapy machines are used. An experiment using kilovoltage and megavoltage energies would therefore show differences in protection.

3. The protective effect may be related to the time between the drug administration and radiation. Studies which vary the times could show different DMF's at specific end points.

4. Different routes of administration of the drug could possibly show different protection against the same radiation reaction. Comparison between oral, intraperitoneal, topical, and intravenous administrations should be made.

5. Finally, since this study suggests that the salivary gland may be another organ of excretion of WR-2721, further radiolabelled WR-2721 studies should be made to help trace the path of the drug administered systemically.

As the information about WR-2721 increases, it suggests that it would be of value in clinical radiation therapy for the protection of normal tissues from the damaging effects of radiation.
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