A Comparative Study on the Protection Profile of Lidocaine, Amifostine, and Pilocarpin on the Parotid Gland during Radiotherapy

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Abstract
The aim of this study was to evaluate the individual and the synergetic radioprotective effect of lidocaine, amifostine, and pilocarpin on the parotid gland. Forty-nine rabbits were randomized into seven groups (n = 7)—control, irradiated sham-treated, irradiated/lidocaine–pretreated, irradiated/amifostine–pretreated, irradiated/pilocarpin–pretreated, irradiated/lidocaine + pilocarpin–pretreated, and irradiated/amifostine + pilocarpin–pretreated groups. One week before irradiation (15 Gy) and 72 hours as well as 1 month afterward, the parotid gland was investigated morphologically, sialoscintigraphically, and immunohistochemically with the use of tenascin-C and α smooth muscle actin. Compared with control animals, there was a significant reduction of the salivary ejection fraction in the irradiated untreated group 72 hours following radiation. Only animals pretreated with lidocaine or amifostine (alone or combined with pilocarpin) showed a slight nonsignificant reduction of salivary ejection fraction. Immunohistochemically, we observed a significant loss of α smooth muscle actin and an up-regulation of tenascin-C expression in irradiated/untreated glands. These changes were less evident in animals pretreated with lidocaine or lidocaine + pilocarpin. Amifostine and pilocarpin did not show any influence on tenascin-C or α smooth muscle actin expression. Ultrastructural damage was observed in irradiated untreated and pilocarpin–pretreated glands. In contrast, lidocaine and amifostine could largely preserve the glandular ultrastructure. One month postradiation, all changes were regressive regardless of treatment protocol. Potential radio-protective agents show different effects on both morphology and function of the parotid gland. Associated immunohistochemo-chemical and ultrastructural findings could prove the pre- vailed protection profile of lidocaine. This may provide a prophylactic approach in the field of radioprotection of salivary glands. (Cancer Res 2005; 65(22): 10486-93)

Introduction
Xerostomia (dry mouth) is a common symptom following radiotherapy of head and neck malignancies, which indicates diminished salivary gland output. Although the degree of salivary gland dysfunction and related p.o. complications is influenced by individual patient characteristics, such as inherent salivary gland activity, age, and sex (1, 2), the most important role is played by the volume of irradiated salivary gland tissue (3). It is known that the parotid gland represents the most radiosensitive salivary gland, causing significant xerosmia if more than half of both glands were irradiated (4). Noteworthy is that saliva reduction becomes manifest during the first week of conventional fractionated radiotherapy (usually after 10-15 Gy) and persists during the rest of the patient’s life (5, 6).

To avoid this distressing side effect, many therapeutic procedures have been introduced to increase the tolerance of the parotid glands during radiotherapy, including either depletion of secretory granula by muscarinic M3 receptors agonists (7) and isoproterenol (8) or recently by pretreatment with the free radical scavenger amifostine (9). A single clinical trial on a further protection of radiogenic sialadenitis and mucositis by coumarin/troxerutine was also reported by Grotz et al. (10). Among the so-called preemptive drugs, pilocarpin has gained ground, not only in experimental studies (11) but in clinical trials as well (12, 13). Postulating the hypothesis of basolateral membrane damage as an initial target site of radiation, Stephens et al. (14) suggested a further prophylactic approach, which has been proven to reduce this damage in cell culture, namely the membrane stabilization agent lidocaine. Besides the sialoscintigraphic changes (15), early immunohistochemical changes such as α smooth muscle actin reduction (loss of myoepithelial differentiation) and tenascin-C matrix remodeling have been described recently following radiation and have proven correlation with functional impairment (16, 17), indicating that initial damage of endpiece epithelium could be identified immunohistochemically.

The aim of the current study was to investigate the potential radioprotective effect of lidocaine, amifostine, and pilocarpin and the synergetic effect of lidocaine/pilocarpin as well as amifostine/pilocarpin on the parotid gland. As variables to evaluate the grade of damage or protection, we used functional sialoscintigraphy, histologic, immunohistochemical, and ultrastructural investigations.

Materials and Methods
Animals and study design. Forty-nine healthy female New Zealand rabbits of 2.5 to 3.5 kg weight were used for the study. They were purchased from Charles-River-Wiga (Sulzfeld, Germany) and kept under laboratory conditions and alternating 12 hours day/night rhythm. All animals were acclimatized for at least 2 weeks before starting the study and then randomized into seven groups (n = 7)—control (sham-treated/unirradiated), irradiated/sham-treated, irradiated/lidocaine–pretreated, irradiated/amifostine–pretreated, irradiated/pilocarpin–pretreated, irradiated/amifostine + pilocarpin–pretreated, and irradiated/amifostine + pilocarpin–pretreated.
groups. All experimental procedures were approved by the local authority according to the current German law on the protection of animals.

Animals underwent a first scintigraphy as described below. Except for the control group, all rabbits were irradiated 1 week later with a single dose of 15 Gy. Pretreatment groups received the drug before irradiation according to the related pharmaceutical features of each substance. Both procedures were done under general anesthesia using a combination of 3 mg/kg (S)-ketamine hydrochloride (Ketanest-S) and 0.1 mg/kg xylazine hydrochloride (Rompun). Seventy-two hours after irradiation, a second scintigraphy was done with a subsequent biopsy of the unilateral parotid gland for immunohistologic and ultrastructural examination. The second scintigraphy was carried out 30 days after irradiation along with a subsequent biopsy of the contralateral parotid gland.

Drugs. Lidocaine hydrochloride (Xylocain, 2%, 1 mg/kg) was slowly administered i.v. 10 minutes before irradiation via a marginal ear vein. Amifostine (Ethylol) was administered according to the recommended dose of 200 mg/m² 15 minutes before irradiation via a marginal ear vein as well. Pilocarpin hydrochloride (Pilomann, 1% EDO) was administered i.p. (4 mg/kg) 90 minutes before irradiation.

Irradiation. Under general anesthesia, X-ray irradiation was done using MEVATRON 74-Siemens teletherapy unit (photon energy) operated at 10 MeV with a dose rate of 3 Gy/min. The output of the accelerator was 1 Gy = 80 MU at source-to-skin distance of 98 cm. The rabbit was placed laterally and covered with a 1-cm-thick tissue equivalent bolus material. An axial beam was directed toward the head of the rabbit, extending from the retroauricular region into the tip of the nose (field size 7.5 × 10 cm), thus including all potential localization of salivary gland tissue. A single dose of 15 Gy was applied by two opposing X-ray tubes. Dose rate was determined using a thermoluminescence dosimeter.

Tissue preparation and immunohistochemistry. The biopsy was always provided from the mediocaudal portion of the parotid gland as previously described (15, 16); the volume of tissue was kept at a minimum (5 × 5 × 5 mm) to avoid injury of major blood vessels, nerves, or excretory ducts, which may, in turn, manipulate subsequent scintigraphic results due to neuronal and vascular impairment or secretory retention. Samples were then fixed in neutral phosphate-buffered 4% formalin. After a minimum of 48 hours of fixation, the tissue was trimmed and processed by standard paraffin-embedding methods. Sections were cut at 4 μm, deparaffinized, and then stained with H&E to obtain conventional histologic sections.

For immunohistochemical staining, the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique was used to visualize the primary antibodies, namely monoclonal mouse antibodies directed against α-smooth muscle actin (clone 1A4, diluted 1:90, DakoCytomation, Glostrup, Denmark) and monoclonal mouse anti-human tenascin (BC4; Dr. L. Zardi, University of Genoa, Genoa, Italy). The APAAP technique was used for visualization of the bound primary antibodies. The secondary rabbit anti-mouse antibody and the APAAP complex were diluted 1:50 (both from DakoCytomation). Naphthol-AS-biphosphate (Sigma, Seelze, Germany) and new fuchsin (Merck, Darmstadt, Germany) were used as substrate and developer, respectively. As negative control, the primary antibody was replaced by a nonimmune serum.

To evaluate the degree of α smooth muscle actin loss per acinus in irradiated glands, a semiquantitative score was introduced to describe the relative decrease of immunostaining around the acini compared with the labeling pattern in the control group. The score was defined as follows: 0, no α smooth muscle actin loss (no difference to control glands); +, 0% to 25% loss of α smooth muscle actin; ++, significant α smooth muscle actin loss >50%; +++, >75% α smooth muscle actin loss.

For the evaluation of tenascin-C reaction, 10 view fields of each sample in every group were chosen at random and the area of positive reaction in relation to the whole view field was calculated using the professional Soft Imaging System software ANALYSIS. Values were expressed as mean ± SE. Areas without acinar cells (fatty tissue) were not considered for evaluation.

Transmission electron microscopy. Samples were immediately fixed by immersion in 0.1 mol/L cacodylate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde (pH 7.4) for 24 hours. The specimens were postfixed in 1% OsO₄ and stained en bloc with 2% uranylacetate. After dehydration in graded alcohols, the specimens were embedded in Araldite. Semithin sections were stained with methylene blue and azure II to visualize the regions of interest containing acinar and myoepithelial cells as well as glandular ducts. Ultrathin sections were cut and stained with lead citrate and examined under an electron microscope (EM 109, Zeiss, Oberkochen, Germany). The findings were recorded both by conventional films and a digital imaging system.

To evaluate the ultrastructural alterations induced by radiation, the following variables were assessed: intracellular edema, nuclear alterations (size and distribution pattern of chromatine), alterations of cytoplasmic organelles (swelling, disintegration, and dissolution), alterations of secretory vesicles (size, shape, and electron density), and the degree of secretion congestion. The findings were recorded by a semiqualitative score as follows: 0, normal; +, minor alterations; ++, moderate alterations; +++, major alterations.

Salivary gland scintigraphy. After i.v. application of 100 MBq ⁹⁹mTcO₄⁻, the rabbits underwent a sequential scintigraphy in a prone position and basal projection of the head, using a single-head γ camera (Picker CX 250 compact, LEHR collimator, field-of-view 25 cm). At the 20 minutes, 0.01 mg/kg Carachol was applied s.c. to stimulate saliva secretion and the scintigraphy was continued for a further 25 minutes. Dynamic studies were acquired with 90 frames and 30 seconds per frame in 256 × 256 matrix with zoom 4. Percentage uptake of the administered activity was calculated for the 10 to 19 and 36 to 45 minutes by summation of the appropriate frames and regions of interest of the parotid glands. Time-activity curves were registered and analyzed.

The second scintigraphy was always done 1 week after the first one to avoid any interaction or uptake disturbance by the radiotracer injected previously.

The salivary ejection fraction has been defined as the activity excreted with the radiolabeled saliva of the parotid gland after Carbachol stimulation expressed as percent: salivary ejection fraction (%) = maximal uptake (till 20 minutes) – remaining activity (after 45 minutes) / maximal uptake of the gland.

Data and statistical analysis. Both parotid glands were evaluated in every animal scintigraphically and immunohistochemically. When proved to follow a normal distribution, values of salivary ejection fraction and tenascin-C reaction were expressed as mean ± SE and the two-sided t test for paired/unpaired samples was used to compare data obtained prior and 72 hours as well as 1 month after irradiation. The Kruskal-Wallis test for unpaired samples was used to evaluate the difference of immunohistochemical reaction of α smooth muscle actin among groups. An α level of P < 0.05 was considered to be significant. Electron microscopic evaluation was only descriptive and underwent no statistical test.

Results

Scintigraphic data. Before irradiation, the initial tracer uptake of the parotid gland in all groups ranged from 0.08% to 0.16% of applied activity. After secretion stimulation with carbachol, the remaining activity decreased significantly from 0.03% to 0.14%. The calculated salivary ejection fraction for the parotid gland before irradiation was 38.28 ± 11.7%.

Three days following radiation of control glands, the primary tracer uptake decreased and the ability of excretion after carbachol administration and thus salivary ejection fraction underwent a significant reduction up to 19.11 ± 12.93% (irradiated sham-treated group; P = 0.03). No significant difference could be observed between the salivary ejection fraction prior and 72 hours following irradiation in animals pretreated with amifostine alone (P = 0.78), indicating intact salivary gland function. Compared with the amifostine group, glands pretreated with lidocaine showed a lower grade of protection after 72 hours (salivary ejection fraction preradiation = 34.38 ± 10.65%; salivary ejection fraction post-irradiation = 28.05 ± 18.34%). However, there was no significant reduction of salivary ejection fraction (P = 0.16). Animals
pretreated with pilocarpin alone displayed a similar reduction of salivary ejection fraction like irradiated sham-treated animals \( (P = 0.02) \). In the combination groups, lidocaine/pilocarpin as well as amifostine/pilocarpin, we could observed a preservation of the salivary ejection fraction \( (P = 0.77 \text{ and } P = 0.15, \text{ respectively}) \). There were no significant differences of the primary tracer uptake after 72 hours among irradiated pretreated glands regardless of the drug(s) administered (data not shown).

One month after radiation, the primary uptake and the salivary ejection fraction of all groups, including the irradiated sham-treated group, met normalization and values measured before irradiation were not significantly different (Table 1).

**Overall histomorphology.** Compared with control glands, the main feature observed in irradiated groups was a secretory retention and an intracellular edema with a consequent rupture of cell membrane especially on the luminal side. The cellular borders seemed diminished and it was not possible to delimit adjacent cells. Scattered vacuolopathy and anisonucleosis was noticed in acinar and ductal cells as well. The secretory retention and vacuolopathy underwent a slight regression throughout the observing period in control and experimental groups; however, no evaluateable differences could be assessed among the different therapy groups neither 72 hours nor 30 days following irradiation. The myoepithelial cells were not always distinguishable and their evaluation was not reliable.

**Tenascin-C.** Tenascin-C distribution in unirradiated control parotid gland displayed a circular pattern around blood vessels; in the parenchyma, it was limited to the basal membrane of the intercalated and secretory ducts and some scattered acinar cells. The staining evaluated digitally amounted to 17 ± 8% of view field (Fig. 1A). Irradiated/sham-treated glands investigated 72 hours later showed a remarkable redistribution of the tenascin-C staining. A significant increase in the expression of tenascin-C in the acinar cells was noticed, reaching 82 ± 31% of view field investigated (Fig. 1B). This up-regulation was reduced in irradiated/lidocaine–pretreated as well as irradiated/lidocaine + pilocarpin–pretreated glands \( (21 ± 13\% \text{ and } 26 ± 10\%, \text{ respectively}; \text{Fig. 2A}) \). Amifostine–, amifostine + pilocarpin–, and pilocarpin–pretreated glands showed no significant difference to the irradiated sham-treated glands \( (76 ± 19\%, 80 ± 23\%, \text{ and } 77 ± 19\%; \text{Figs. 2B and C}) \).

The combination with pilocarpin had no significant effect on the tenascin-C redistribution.

We could not notice any difference among samples examined 1 month after irradiation. The glands in all groups displayed normalization and no longer significant differences to unirradiated parotid gland tissue (Table 1).

**α Smooth muscle actin.** In control parotid glands, myoepithelial cells were stained around the acini forming a hem-like figure, whereas the myoepithelial cells of the intercalated and secretory ducts as well as blood vessels displayed a circular distribution (Fig. 1C). Irradiated/sham–treated parotid glands investigated 72 hours after irradiation displayed a special pattern of staining: The myoepithelial cells of the endpieces were irregularly stained and a lot of acinar myoepithelial cells were not stained at all \( (Fig. 1D) \); there was a significant loss of α smooth muscle actin staining in all samples. On the contrary, the myoepithelial cells of the intercalated and secretory ducts remained either unaffected or showed less impairment. Irradiated/lidocaine–pretreated glands showed no significant difference in the expression pattern of α smooth muscle actin to unirradiated glands (Fig. 2D). In contrast, pretreatment with amifostine or pilocarpin did not hinder massive α smooth muscle actin staining.

| Table 1. Scintigraphic and immunohistochemical evaluation of the parotid gland of all study groups before irradiation 72 hours and 1 month following irradiation with 15 Gy |
|---------------------------------|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Preradiation     | After 72 h      | \( P \)         | After 1 min     | \( P \)         |                |                |
| **SEF (%)**                    |                  |                 |                 |                 |                 |                |                |
| Only irradiated                | 38.28 ± 11.7     | 19.11 ± 12.93   | 0.03            | 37.23 ± 6.91    | 0.42            |                |                |
| Lidocaine                      | 34.38 ± 10.65    | 28.05 ± 18.34   | 0.16            | 26.08 ± 16.39   | 0.19            |                |                |
| Amifostine                     | 22.67 ± 11.26    | 21.52 ± 6.65    | 0.78            | 23.06 ± 12.09   | 0.24            |                |                |
| Pilocarpin                     | 32.25 ± 11.54    | 18.46 ± 8.83    | 0.02            | 31.01 ± 9.31    | 0.34            |                |                |
| Lidocaine/pilocarpin           | 21.92 ± 9.41     | 20.85 ± 12.98   | 0.77            | 21.32 ± 9.81    | 0.24            |                |                |
| Amifostine/pilocarpin          | 24.57 ± 12.14    | 11.70 ± 3.50    | 0.15            | 16.10 ± 3.45    | 0.32            |                |                |
| **TN-C (%)**                   |                  |                 |                 |                 |                 |                |                |
| Only irradiated                | 17 ± 05%         | 82 ± 19%        | 0.002           | 18 ± 06%        | 0.09            |                |                |
| Lidocaine                      | 19 ± 02%         | 21 ± 08%        | 0.33            | 20 ± 06%        | 0.57            |                |                |
| Amifostine                     | 15 ± 09%         | 76 ± 19%        | 0.02            | 17 ± 04%        | 0.52            |                |                |
| Pilocarpin                     | 19 ± 07%         | 77 ± 17%        | 0.05            | 19 ± 10%        | 0.55            |                |                |
| Lidocaine/pilocarpin           | 24 ± 06%         | 26 ± 10%        | 0.56            | 26 ± 08%        | 0.71            |                |                |
| Amifostine/pilocarpin          | 18 ± 07%         | 80 ± 20%        | 0.01            | 20 ± 02%        | 0.63            |                |                |
| **ASMA (score)**               |                  |                 |                 |                 |                 |                |                |
| Only irradiated                | 0                | +++             | <0.001          | 0 (+)           | >0.05           |                |                |
| Lidocaine                      | 0                | 0 (+)           | >0.05           | 0 (+)           | >0.05           |                |                |
| Amifostine                     | 0                | ++             | <0.001          | +               | >0.05           |                |                |
| Pilocarpin                     | 0                | ++ (+)          | <0.001          | +               | >0.05           |                |                |
| Lidocaine/pilocarpin           | 0                | 0 (+)           | >0.05           | 0 (+)           | >0.05           |                |                |
| Amifostine/pilocarpin          | 0                | ++ (+)          | <0.001          | +               | >0.05           |                |                |

Abbreviations: SEF, salivary ejection fraction; TN-C, tenascin-C; ASMA, α smooth muscle actin.
loss (Figs. 2E and F). The protective effect of combined therapy was only evident for the lidocaine/pilocarpin group. One month later, no significant changes could be noticed in the parenchyma of various groups; however, loss of acinar cells and associated structures influenced the total labeled areas with α smooth muscle actin (data not shown).

Transmission electron microscopy. The ultrastructural inves-
tigations were done selectively in two glands of each monotherapy group because drug combinations did not influence the outcome of pretreatment.

The glandular acini of untreated-irradiated animals showed a remarkable intracellular edema, electron lucent cytoplasm, and a reduction of cytoplasmic organelles. The secretory vesicles displayed a heterogeneous ultrastructure characterized by the concomitant presence of either electron dense or electron lucent granular material. In addition, several vesicles changed from round to angular shapes and frequently showed multiple intravesicular vacuolations (Fig. 3A).

In contrast, irradiated/lidocaine–pretreated glands revealed a rather normal morphology at ultrastructural level. Neither relevant cellular swelling nor decrease of intracellular organelles was observed. The densely distributed secretory vesicles showed a uniform appearance with round shapes and were filled homoge-
neously with electron dense secretion material (Fig. 3B).

Glandular acini derived from irradiated/amifostine–pretreated animals basically resembled the ultrastructural morphology en-
countered in the lidocaine-pretreated group. However, several acinar cells reproducibly showed intracellular edema and were poorly equipped with cytoplasmic organelles. Moreover, a subset of secretory vesicles lost its round shape, became larger in size, and formed irregular borders (Fig. 3C). These alterations were most likely due to an overproduction of secretion of individual vesicles rather than to a confluence of neighboring vesicles, as different stages ranging from small- to large-sized vesicles could be observed.

In comparison with both the lidocaine- and amifostine-pre-
treated groups, irradiated animals pretreated with pilocarpin...
showed the most severe ultrastructural alterations similar to those observed in untreated radiated animals (Fig. 3A). The acinar glands were characterized by intracellular swelling, paucity of organelles, and secretory vesicles of highly varying electron density. Whereas some areas were filled with normally shaped vesicles of electron dense secretion material, most regions displayed clusters of densely packed vesicles containing granular material of either minor or very poor electron density (Fig. 3D). All findings underwent a

Figure 2. Pretreatment with lidocaine prevents overexpression of tenascin-C (A) as well as \( \alpha \) smooth muscle actin loss (D). In contrast, significant changes in the expression of both antibodies are still evident after treatment with amifostine (B and E) and pilocarpin (C and F).
semiquantitative analysis of the ultrastructural changes described above. The results are summarized in Table 2 and confirm the different degrees of ultrastructural damage characteristics for each experimental group.

Discussion

Salivary gland damage associated with structural alteration and functional restriction is a well-known sequela of radiotherapy in the head and neck region. Apart from partial sparing of parotid gland through three-dimensional planning of radiotherapy (18) and saliva substitution after radiotherapy, many pharmacological approaches have been used, including prophylactic agents or sialogogues. The prophylactic approach became important because of improving compliance of patients and its economic advantage.

To study the effect of potential radioprotective drugs on salivary gland function, reliable variables are required. Besides the evaluation of scintigraphic findings and flow rates, previous studies also considered morphologic aspects (19–21). However, the morphologic criteria applied to estimate the degree of damage or protection were variable. Studies suggesting the use of amifostine in the radioprotection of salivary glands have indicated the grade of subsequent lipomatosis of the parotid gland as a protection variable (19). This finding is, however, attributed to the late regeneration process and thus could not serve as a reliable indicator of radiation damage, particularly when considering that the parotid gland in this experimental model displays naturally extensive fatty tissue distribution. Other studies have indicated glandular weight gain and reduction of p.o. complications as a sign of radioprotection (22). Morphologic signs reported as an indicator of salivary gland protection using pilocarpin were also rare and unspecific. They ranged from weight loss of the studied gland to acinar cell loss (23, 24). The only report regarding the use of lidocaine for salivary gland protection was derived from Stephens et al. (14), who have

Table 2. Semiquantitative evaluation of the ultrastructural changes of the parotid gland tissue in the monotherapy groups 72 hours following single dose irradiation with 15 Gy

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cellular Edema</th>
<th>Nucleus Organelles</th>
<th>Cytoplasmic Vesicles</th>
<th>Secretion Congestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lidocaine</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Amifostine</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pilocarpin</td>
<td>+++</td>
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<td>+++</td>
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NOTE: 0, normal; +, minor alteration; ++, moderate alteration; +++, major alteration.

Figure 3. Transmission electron microscopy of glandular acini from irradiated parotid glands without pretreatment (A) compared with irradiated glands pretreated with lidocaine (B), amifostine (C), and pilocarpin (D). A, acinar cells are characterized by intracellular swelling, paucity of cytoplasmic organelles (*), and secretory vesicles containing secretion material of different electron density (arrows). Several vesicles display angular shapes and multiple vacuolations (arrowheads). B, acinar cells are densely packed with normally configured secretory vesicles and reveal no major morphologic alterations at ultrastructural level. C, acinar cells show moderate intracellular edema (asterisk). Although most of the secretory vesicles are normally formed, a subset of vesicles is irregularly shaped and larger in size (arrows). D, acinar cells are characterized by remarkable intracellular swelling, decrease of intracellular organelles, and secretory vesicles of highly varying electron density ranging from electron dense to electron lucent granular secretion material (arrows). Bar, 3 μm.
considered the low grade of nuclear aberration as the goal effect of pretreatment. Apart from earlier investigation assessing the capability of local anesthetics from the amid group to stabilize and protect the cell membrane during radiation in cell cultures (25, 26), the use of lidocaine as a radioprotective agent of the salivary gland is uncommon and has only been used in a single study on acinar cell culture (14).

In the present study, we investigated the radioprotective effect of amifostine, lidocaine, and pilocarpin as well as the combination of amifostine + pilocarpin and lidocaine + pilocarpin in an established rabbit model. The aim was to evaluate the potential synergic effect of substances with direct influence on the stabilization of cell membrane and structures like amifostine or lidocaine (8, 9, 22, 25, 26) with the widely examined muscarinic alkaloid pilocarpin, which induces depletion of secretory granules in acinar cells, a process that was considered radioprotective in previous studies (7, 11, 12, 20). To provide comparable data, we used a single-dose irradiation of 15 Gy, the radiation modality used by the majority of experimental studies regarding radiation-induced impairment and protection of salivary glands (7, 11, 15, 20, 21, 24). Further studies dealing with the role of fractionation at the histomorphologic and functional level gave evidence on the negligible protective effect of this modification on the morphology of acinar cells at this stage. According to these studies, irradiation with 15 Gy given in a single dose would implicate acinar cell impairment similar to that observed using conventional or accelerated fractionation (6, 21, 27).

Using this experimental model, we could show that the use of lidocaine in the dosage recommended for interrupting ventricular fibrillation (1 mg/kg) applied 15 minutes before irradiation could reduce the functional impairment and avoid reduction of salivary ejection fraction. The limited expression of tenascin-C observed in samples investigated 72 hours after irradiation could be attributed to the direct stabilization effect of lidocaine on the acinar cell membrane and extracellular matrix interaction. Immunohistologic data derived from previous studies may explain the relationship between cell membrane damage and up-regulated tenascin-C expression in both acinar and myoepithelial cells (16, 17, 28, 29), an aspect that was evident in our results through α smooth muscle actin preservation. The most efficient preservation of the ultrastructural morphology of irradiated glandular tissue was also achieved by administration of lidocaine. Both the general cellular integrity and the structural configuration of the secretory apparatus were not essentially altered when compared with unirradiated glands. The fact that local anesthetics are able to sensitize the cell to radiation damage when present after irradiation indicates the overall course of such histologic and functional changes had, however, no effect on the secretory function of the gland. To explain these results, we suggest a threshold of minimal cell membrane damage that may induce α smooth muscle actin and tenascin-C remodeling without implicating salivary function damage.

The use of pilocarpin to improve the secretory function of impaired salivary glands after radiotherapy is a common approach and already in clinical use (34). Clinical and experimental trials on the prophylactic effect of muscarinic receptor agonists, however, provided controversial data. Whereas pilocarpin reduced structural damage and weight loss in a rat model (20, 21), clinical trials showed no significant protective effect (12). Our immunohistologic and ultrastructural results showed moderate cell damage and signs of myoepithelial cell disintegration after application of pilocarpin. The cellular alterations were more pronounced at ultrastructural level, in particular compared with both lidocaine and amifostine groups. The evidence of electron lucent granules, such as those observed in sialadenitis, most likely reflects alterations in the protein consistency of secretion (35, 36). The up-regulation of tenascin-C in the acinar cells 72 hours following radiation may also indicate early subbasement membrane alteration. This hypothesis is strongly supported by our scintigraphic results, which displayed disturbance of primary $^{51}$Co uptake as well as reduction of salivary ejection fraction. Normalization of scintigraphic and immunohistologic findings observed at 1 month including all groups may indicate functional recovery. These results are in accordance with several clinical trials, which registered a threshold of 22 to 26 Gy as critical dose for the parotid gland (37–39). Potential changes between the two investigation time points are possible and could be revealed by an alternative study design. The interval chosen, however, provides comparable data and showed significant changes that indicate the overall course of such histologic and functional changes of salivary glands.

Considering all this, the prophylactic application of lidocaine and amifostine seems to reduce functional, immunohistochemical, and structural damage of the parotid gland during radiotherapy. Pilocarpin may represent an adjuvant approach postradiation to stimulate survived acinar cells. Taking into consideration that lidocaine has not yet been used clinically for this purpose and the pharmacokinetic of amifostine is still widely unknown, further experimental studies are needed to rule out potential protection of tumor cells. Keeping in mind that the encouraging results presented above, although being evidential are valid for the parotid gland in a rabbit model, clinical trials are crucial to introduce a comprehensive and effective procedure for protecting salivary glands during radiotherapy of the head and neck region.

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