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Late effects

Optimum dose range for the amelioration of long term radiation-induced hyposalivation using prophylactic pilocarpine treatment

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Abstract

***Background*: To determine dose and time dependency of pilocarpine pre-treatment protection from late damage after unilateral irradiation of the rat parotid gland.**

***Methods and materials*: The right parotid gland of saline (1 mg/ml) or pilocarpine (4 mg/kg) pre-treated rats was irradiated with 10, 15 and 20 Gy. Saliva was collected from the irradiated and shielded parotid before, 30, 60, 120 and 240 days after irradiation. The number of acinar cells/gland was determined 30, 120 and 240 days after irradiation by histological examination.**

***Results*: Pilocarpine pre-treated rats, protection of parotid gland function was seen in the early-intermediate phase (0—120 days) after 15 Gy and in the late phase (>120 days) after 10 and 15 Gy. Although no protection was observed after 20 Gy, a stimulatory effect of pilocarpine on the non-irradiated gland resulted in a significant increase in total saliva secretion.**

The increase in function after pilocarpine treatment was paralleled by a significant increase in the number of acinar cells in both the irradiated and shielded glands.

***Conclusions*: Pre-irradiation treatment with pilocarpine induces compensatory response, at lower doses, in the irradiated and at higher doses in the non-irradiated gland reducing late damage, due to stimulation of unirradiated or surviving cells to divide.**
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Keywords: Pilocarpine; Parotid gland; Radiation-induced damage; Radioprotection

The exposure of salivary gland to irradiation during radiotherapy among patients with head and neck cancer often results in hyposalivation [1]. Hyposalivation is one of the major causes for the development of radiation-induced xerostomia (dry mouth syndrome) which has a negative impact on the quality of life of patients [2]. The mechanisms resulting in radiation-induced hyposalivation in rodents are beginning to emerge [3], and have revealed a number of potentially interesting treatment approaches to prevent radiation-induced hyposalivation [4]. Experimentally, the tolerance of the rodent parotid gland against ionizing radiation has been successfully enhanced using sialogogues [5], radical scavengers [6—9] and recently also with a membrane stabilizing agent [8]. Effective amelioration of the early effects can be achieved using prophylactic treatment with sialogogues like pilocarpine [10—12], a drug that also clinically can be applied with mild side effects [4]. Moreover, the results of a double-blind randomised, placebo-controlled study suggested that concomitant administration of pilocarpine during radiotherapy resulted in sparing of late radiation effects on parotid gland function in glands radiated with a mean dose above 40 Gy [13].

Despite these promising results, we recently showed in rats that the radioprotective effects of pilocarpine pre-treatment diminish with dose and time after radiation when salivary glands are completely within the radiation field [12]. However, in the clinical setting, full salivary gland irradiation will only occur rarely, in particular when radiation techniques that enable significant sparing of the glands, like 3D-conformal radiotherapy and IMRT, have been implemented [14,15] and result in a considerable sparing reduction but not obliteration of side effects [16,17].

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Interestingly, it has been hypothesized that increasing the compensatory potential of the non-damaged gland, at least in part, underlies the early ''radioprotective effect'' of pilocarpine in case of unilateral radiation [11]. This suggests that the ability of pilocarpine to ameliorate the radiation-induced impairment of the parotid gland function may depend on the remaining number of functional cells, and thus to the volume of the gland that lies outside the radiation portals and the radiation dose [11]. By stimulating muscarinic-acetylcholinergic receptors, pilocarpine seems to stimulate functional cells that survived the radiation insult or that are located outside the radiation portal to compensate for the loss of function of the radiation-damaged cells. When the salivary glands are all completely irradiated to a high dose, the number of surviving cells able to compensate may be too low to induce long lasting protection [12].

The hypothesis that the ability of pilocarpine pretreatment to ameliorate late irradiation-induced hyposalivation depends on the amount of non-damaged cells in the tissue, and thus is dose and volume dependent, was further tested in this study.

Materials and methods

Animals

Male, 8—9 weeks old (body weight 260—280 g) albino Wistar rats of strain Hds/Cpb: WU (Harlan CPB, Rijswijk, The Netherlands) were used. They were housed in polycarbonate cages (six rats per cage) under a 14:10-h light:dark cycle. The rats were kept in the experimental unit for 1.5 weeks prior to the experiments. Food (RMH-B, Hope Farms, Woerden, the Netherlands) and water were given ad libitum. All experiments were performed in agreement with The Netherlands Experiments on Animal Act (1977) and the European Convention for the Protection of Vertebrates Used for Experimental Purposes (Strasbourg, 18.III.1986).

Radiation procedure

Prior to irradiation all rats were anaesthetized by an intraperitoneal injection of Ketalar 60 mg/kg and Rompun 2.5 mg/kg. A 6-mm-thick lead shield with a tailor-made portal was positioned so as to permit direct unilateral, right, parotid gland irradiation. Most of the right submandibular/sublingual and the complete left submandibular/sublingual and left parotid region and oral cavity were excluded from the treatment portal [12]. Meanwhile the rest of the body, including the oral cavity, was shielded. This setup prevents other disorders than the ones inflicted on the salivary glands and ensures an optimal nurturing status of the rats post-irradiation. The gland area was irradiated with a single dose of 10, 15, and 20 at 1.5 Gy min^{-1}. The X-ray apparatus (Mueller MG 300, Philips, Eindhoven, The Netherlands) was operated at 15 mA, 200 kV (filters 0.5 mm copper, 0.5 mm aluminium; HVL = 1 mm copper). Dose rate was determined in air with a calibrated electrometer and ionization chamber combination (Keithleg 35040 + NE 2571).

Treatments

One hour prior to irradiation rats were given prophylactic treatments being:

1. Saline (1 mg/ml) and irradiated with a single dose of 10, 15 or 20 Gy
2. i.p. 4 mg/kg pilocarpine and irradiated with a single dose of 10, 15 or 20 Gy

Seventeen rats per dose treatment group, 8 for function measurement and 9 for morphology assessment, were used.

Collection of saliva

Saliva samples of both left and right parotid gland were collected separately and simultaneously under isoflurane/O_2 anaesthesia by means of miniaturized Lashley cups [18]. The cups were placed upon the orifices of both parotid glands. Saliva was collected for 30 min after stimulation with 2 mg kg^{-1} pilocarpine ((+)-Pilocarpine hydrochloride and sodium pentobarbital, Pharmacist University Medical Center Groningen, The Netherlands) administered subcutaneously (at t = 0 and t = 15 min). Saliva was collected in pre-weighed ice-cooled plastic tubes 4 days before and 30, 60, 120, 180 and 240 days after irradiation. The total volume of saliva secreted was estimated by weight, assuming the specific gravity of saliva to be 1.0 g cm^{-3}. The saliva flow rate (μl min^{-1}) was calculated from the collecting time and volume, and expressed as % of the value before irradiation (±SEM).

Tissue preparation and observation methods

At 30, 120, and 240 days after irradiation 3 rats were prepared for tissue examination. They were anaesthetized with an i.p. injection of pentobarbital (60 mg/kg body weight) and exsanguinated, thereafter the right and left parotid gland were taken out carefully and weighed. The tissue was fixed by immersion in 4% formaldehyde/PBS for 24 h at room temperature. A standard graded alcohol procedure was used to dehydrate the glands. The tissues were embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Sections of 2 μm were cut with a Leitz microtome (Wetzlar type 1212, Germany). The sections were stained with haematoxylin and eosin (H&E). Each gland was individually examined at three different levels with a distance of 100 μm. At each level, five fields with an area of 0.034 mm^2 (0.185 mm $\times$ 0.185 mm) and at a magnification of 400$\times$ were randomly chosen and investigated. In each field the number of acinar cells was scored. Subsequently, the average was multiplied by the gland weight of that rat and divided by the gland weight of control animals. Thus a value in arbitrary units proportional to the absolute number of acinar cells per gland was obtained. The number of cells for controls was set to 100%. The values for the other conditions were expressed as a percentage (±SEM) of this control.

Statistical analysis

The changes observed were expressed as a percentage of the pre-treatment. To evaluate early (0—120 days) or late (120—240 days) effects the areas under the curve for 0—120 or 120—240 days, respectively, were calculated (Fig. 1d) and expressed as the percentage change compared with sham-irradiated non-treated controls. The area under the curve was calculated using the percentage of function (flow rate) of number of acinar cells, as the ordinate and

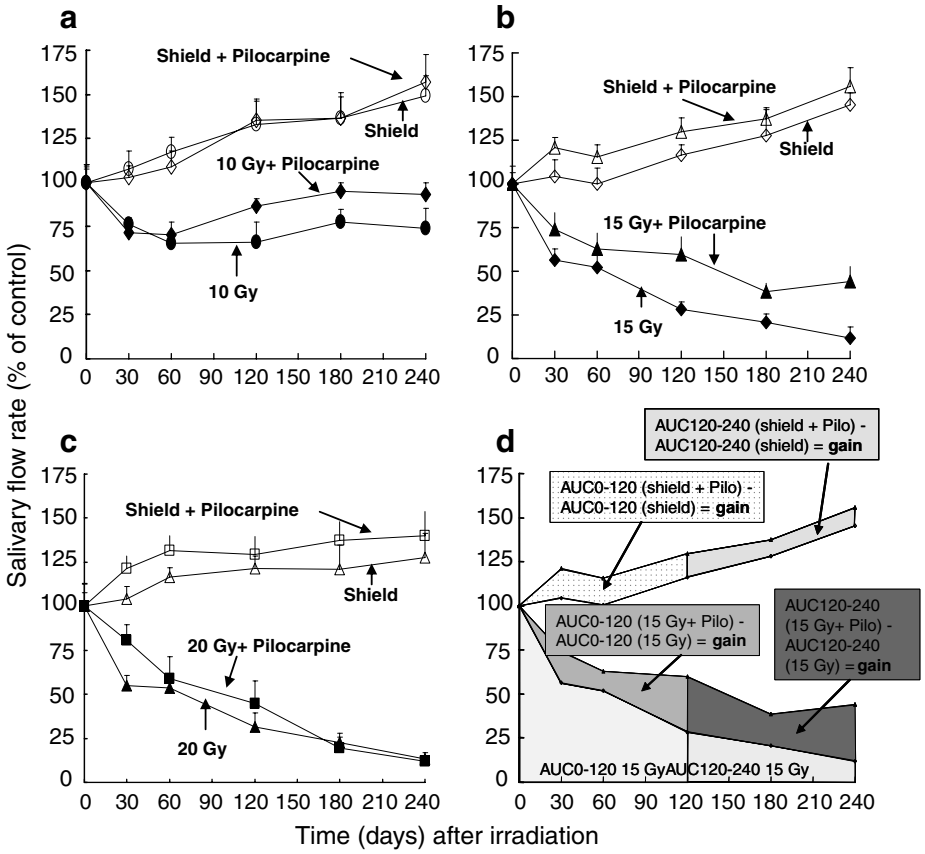

Fig. 1. Long term effects of prophylactic pilocarpine treatment on salivary flow rate of irradiated (solid symbols) and shielded contralateral (open symbols) parotid glands. (a) 10 Gy, (b) 15 Gy, (c) 20 Gy, (d) 15 Gy + AUCs. Flow rate is depicted as ±SEM of pretreatment values. $N = 8$, $^{*}p < 0.05$, for whole curve.

the time (days) as the abscissa (Fig. 1). The results are expressed as means ± SEM. The results were analyzed using a Mann–Whitney test.

Results

First, we examined the long-term effect of prophylactic pilocarpine treatment on the function of unilaterally irradiated parotid glands. Radiation caused a dose dependent decrease in the irradiated parotid gland flow rate with no recovery of function up to 240 days post-radiation when the dose exceeded 10 Gy (Fig. 1a–c). The shielded parotid gland increased in function to an extent expected when the normal growth of the gland is taken into consideration [12,19]. At 15 Gy, pilocarpine provided late protection of the irradiated gland (Fig. 1b, closed symbols), which differs from our earlier findings of a lack of late protection after bilateral irradiation with this dose [12]. In accordance with our hypothesis, the shielded gland also showed a gain in flow rate after pilocarpine treatment that was significantly ($p < 0.05$) higher than the gain seen in the shielded gland in animals that were not pre-treated with pilocarpine (Figs. 1b and c, open symbols).

To be able to compare the protective effect of pilocarpine over the different doses for irradiated and shielded glands in time, the area under the curve (AUC) for the sham and pilocarpine-treated animals over the first 120 days (early-intermediate phase [12]) and 120–240 days (late phase) after irradiation was determined. By deduction of the sham-treated AUC from the AUC of the pilocarpine-treated group, a quantitative estimate of the total gain in saliva flow rate could be determined (see Fig. 1d for an example of the 15 Gy group). For the early-intermediate phase (0–120 days), a significant improvement of salivary flow after pilocarpine treatment was only found when the parotid glands were irradiated with 15 Gy (Fig. 2a). For the late period, a significant protective effect of pilocarpine was found when they were irradiated with 10 and 15 Gy (Fig. 2b). At 20 Gy no significant pilocarpine-induced protection was seen anymore (Fig. 2a and b). This is consistent with the suggested dose dependency of pilocarpine as a ''radioprotective'' agent. Stimulatory effects of pilocarpine on the shielded gland were observed when the *shielded* gland was irradiated with the high dose of 20 Gy for both phases (Fig. 2, open bars). This effect is more pronounced at higher doses in the late phase. When the irradiated gland functions better, less compensation of the shielded gland seems to occur. Prophylactic pilocarpine further increases this effect (Figs. 1b and c) although functional compensation of shielded glands normally also occurs. When the combined salivary flow is assessed, a clear protective effect on total saliva output is observed for both phases (Fig. 2).

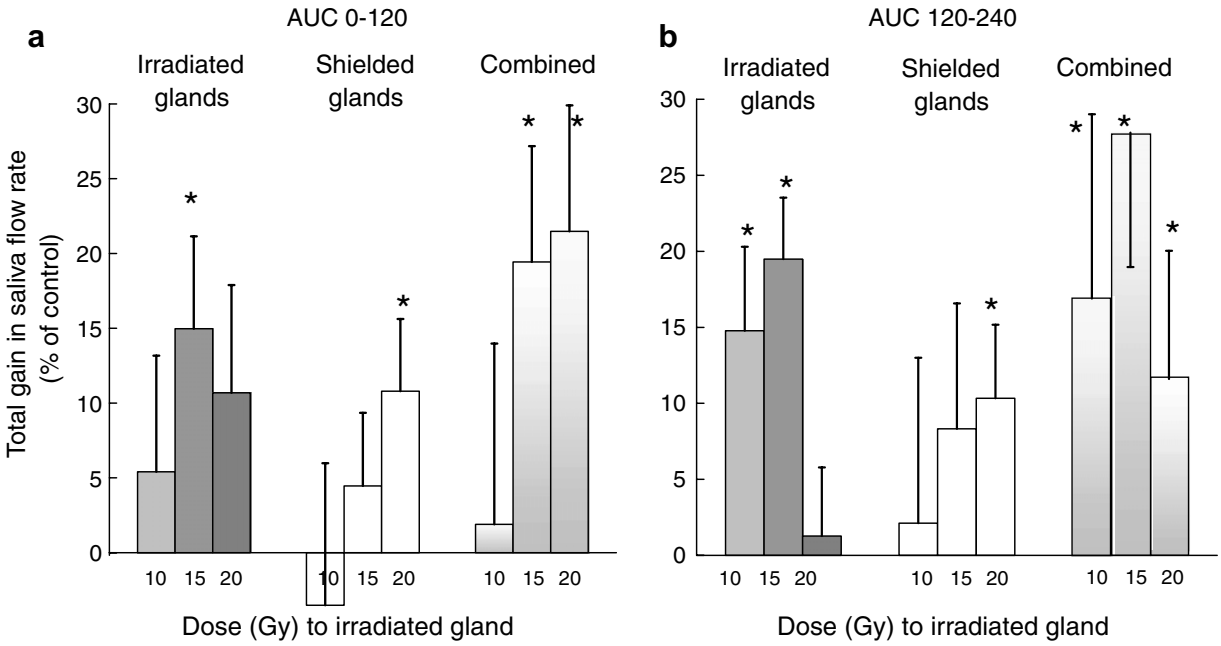

Fig. 2. Dose dependent gain in salivary flow rate due to pilocarpine pretreatment. Filled bars represent irradiated gland, open bars represent shielded glands and shaded bars represent the combined gain in salivary flow of the shielded and irradiated glands. Difference between the AUC of the curves of treated and untreated glands and depicted as % of unirradiated controls. $N = 8$, $^*p < 0.05$.

The compensatory responses in the shielded glands induced by pilocarpine suggested that pilocarpine may stimulate resident salivary gland stem cells to divide and to differentiate into functional acinar cells. To test this idea, the number of acinar cells per gland was determined at days 30, 120 and 240 after irradiation. Radiation caused a dose and time dependent decrease in number of acinar cells in the irradiated parotid gland, being most prominent at 240 days after radiation. At this time point some recovery of the acinar cell number was seen after a single dose of 10 Gy (Fig. 3), in agreement with a functional recovery of the gland at this time point. The acinar cell counts in the shielded glands increased in time, consistent with age-related growth of the glands (Fig. 3, open symbols). Interestingly, when the animals were irradiated to the contralateral with 20 Gy, the number of acinar cells increased significantly ($p < 0.05$) in the shielded gland at an earlier time point (day 30) after radiation. This effect equalled out in time to yield the same numbers at 240 days after all radiation treatments. Like the data on gland function, the area under the curve (AUC) for the sham and pilocarpine-treated animals over the first 120 days (early-intermediate phase [13]) and 120—240 days (late phase) after irradiation was determined. By subtraction of the

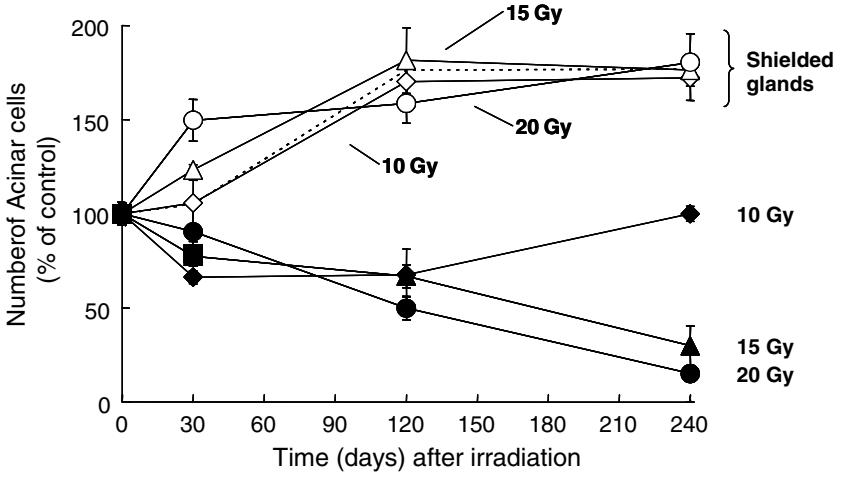

Fig. 3. Compensatory response in shielded glands. Number of acinar cells as % of unirradiated controls at the time of irradiation. Closed symbols are the irradiated glands, open symbols are shielded glands. Acinar cell number is calculated from the amount of acinar cells per high power field × irradiated gland weight/control gland weight. $N = 3$.

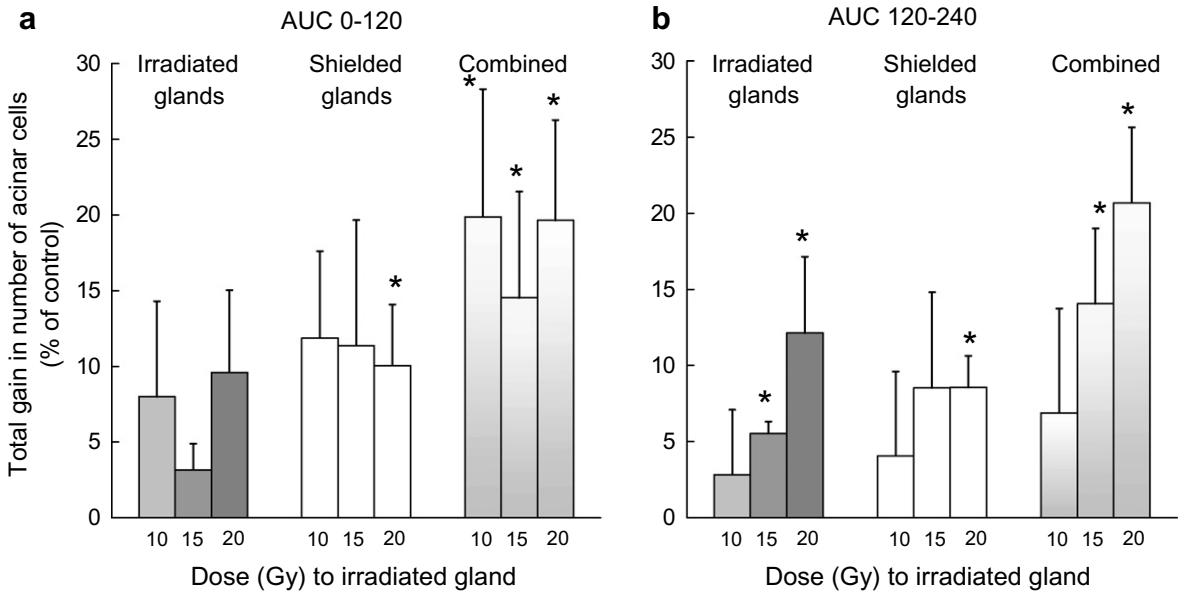

Fig. 4. Dose dependent gain in acinar cell number due to pilocarpine pretreatment. Filled bars represent irradiated gland, open bars represent shielded glands and shaded bars represent the combined gain in salivary flow of the shielded and irradiated glands. Difference between the AUC of the curves of treated and untreated glands and depicted as % of unirradiated controls. $N = 8$, $^{*}p < 0.05$.

sham-treated AUC from the AUC of the pilocarpine-treated group, like the analysis performed for the flow rates, a quantitative estimate of the total gain in acinar cell number was determined.

Adding pilocarpine increased the number of acinar cells only significantly in the irradiated gland late after irradiation and only after 15 and 20 Gy (Fig. 4). Early to intermediate after irradiation, the shielded glands clearly showed an increase in acinar cell number, which was significant only after 20 Gy to the irradiated gland. During the late phase pilocarpine induced an increase in the number of acinar cells, again being significant after 20 Gy. The combined number of acinar cells increased at all doses and time points.

In general, the gain in number of acinar cells followed the gain in saliva flow rate after pilocarpine treatment. Furthermore, the highest sparing effect of pilocarpine was observed at 15–20 Gy when the cumulative effect of the irradiated and shielded gland yielded the highest total salivary flow and acinar cell number.

Discussion

This study was initiated to investigate the ability of pilocarpine pretreatment to ameliorate late irradiation-induced hyposalivation and how this depends on the amount of non-damaged cells in irradiated and shielded parotid gland tissue. We showed that a single prophylactic treatment of pilocarpine shortly before irradiation induced a small but significant amelioration of radiation-induced damage to the rat parotid gland. Part of this was due to an extra compensatory response elicited above the normal compensatory response of shielded gland. The level of the compensatory response in the shielded gland seems to be related to the amount of damage inflicted to the irradiated gland.

Therefore, it seems that the protective effect of pilocarpine is mainly due to the stimulation of non-irreversibly damaged stem cells, either surviving the irradiation insult or laying outside the irradiation field.

In earlier studies, it was already suggested that pilocarpine could ''protect'' against the early phase of radiation damage to the salivary gland [10–12,20,21]. The current study also showed late ''protection'' although pilocarpine pretreatment could not prevent further deterioration. The mechanism of protection of pilocarpine may be the stimulation of compensatory responses by stimulation of surviving tissue stem cells remaining after irradiation or present in shielded glands, as was suggested in previous publications [10,11].

Pilocarpine stimulates the musarinic receptors on salivary gland cells which activate the ERK1/2 signaling, involved in a variety of biological effects like differentiation and proliferation [22]. Together with extra nerve input necessary to induce saliva secretion from the few cells left, this may induce undamaged stem cells, which reside in the ductal compartment of the salivary gland, to proliferate and differentiate in acinar cells and repopulate the damaged tissues [23]. The capability of such salivary glands stem cells to repair damaged tissue after radiation has been shown by Lombaert et al. [24], who suggested that bone marrow derived cells when mobilized and homed to the irradiated salivary gland may secrete factors which induce the remaining stem cells to proliferate and repair to a certain extent tissue damage.

Both in animals and humans negative [8,25,26] and positive [5,10,11,21,27–31] results were obtained when pilocarpine was administrated prophylactic. The relatively small protective effect (Maximal about 20% gain in flow) may explain the contradicting results presented in the literature. Our study seems to confirm the hypothesis that the protec-

tive effects of prophylactic pilocarpine are due to stimulation of ''healthy'' tissue in and out-side the irradiation field [4].

In a recent paper, the results of a double blind randomised, placebo controlled study in humans showed that pilocarpine pre-treatment was able to ameliorate late damage to the parotid gland in patients treated with radiotherapy for a head and neck tumour [13]. In this study stratification for the volume of the parotid gland receiving >40 Gy was done before randomisation, to get a wide range in the dose-volume distribution in both groups. A significant difference was observed in the group of patients with a mean parotid dose >40 Gy receiving pilocarpine pre-treatment compared to the placebo group. In the group of patients with a mean dose <40 Gy, the incidence of late xerostomia was low, so differences between the pilocarpine pre-treatment and the placebo groups were difficult to detect. Apparently, a substantial amount of damage had to be present to observe the effect of pilocarpine given during radiation, indicating that after low dose irradiation pilocarpine was not necessary to induce ''repopulation''. On the other hand, the number of patients receiving a mean dose of >60 Gy was very limited. Above this dose it is not likely to see any protective effect. Indeed in most negative studies more than 50% of the glands were irradiated with 50 Gy [25,26]. In these irradiated parotid glands, probably the number of stem cells that was destroyed has exceeded the critical limit, explaining the loss of the protective effect of pilocarpine given during radiotherapy.

In conclusion, when the saliva flow is reduced below a certain threshold, unirradiated cells or cells surviving the radiation insult may be stimulated to divide and yield a compensatory response. This compensatory response is of minor clinical importance when there is no need to provide the gland with extra input since there is no severe lack of saliva. In addition, this compensatory response is also clinically negligible when the dose to the parotid gland tissue has exceeded the critical limit for damage to the stem cell compartment to be able to compensate to a relevant level for the functional radiation-induced loss of parotid gland function. In between these ranges an optimum dose range must exist where pilocarpine elicits its beneficial effect. Further studies are necessary to establish the exact dose range in humans.

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