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ORIGINAL ARTICLE

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Does irradiation affect the protein composition of saliva?

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Abstract The purpose of this study was to compare the relative amount of low molecular weight salivary proteins in patients with head and neck tumours treated with radiotherapy and healthy subjects. Reverse-phase high-pressure liquid chromatography was used for protein separation. Nine protein fractions (including acidic and basic proline-rich proteins (PRPs), cystatins, histatins and statherin) were identified in saliva from irradiated patients as well as healthy subjects. However, compared with non-irradiated healthy subjects, the fraction of acidic PRPs was significantly reduced in irradiated patients. These data indicate an alteration of the relative amount of low molecular weight salivary proteins in irradiated patients besides the reduction of salivary flow.

Keywords Hyposalivation · Saliva composition · Xerostomia · PRPs

Introduction

Radiation-induced xerostomia is one of the most common and prominent long-term side-effects in patients after therapeutic irradiation of tumours in the head and neck region [18]. Xerostomia is the result of irradiation-caused damage of the major salivary glands. Due to this damage, the flow rate of unstimulated saliva is reduced to 5% of the pre-radiation value [23]. Stimulated saliva seems to be more affected than unstimulated, reflecting the incapability of the irradiated glands to increase their production after stimulation [11, 15, 21]. The residual flow rate is significantly related to the dose of irradiation [22].

Apart from the reduction of the salivary flow rate, the quality of saliva undergoes significant changes due to radiotherapy. The saliva becomes highly viscous, and there is a shift of its pH to more acidic values [2, 4, 12]. In addition, it has been reported that the protein concentration of saliva increases during radiation and returns to pretreatment values after cessation of radiation [7, 13, 16]. However, other investigations did not indicate statistically significant changes of the total protein concentration in relation to irradiation [2, 9].

Concerning the effect of irradiation on the protein composition of saliva, contradictory results were also published. Whereas in some studies a shift in the salivary composition with regard to specific proteins, e.g. IgA, amylase, lysozyme or lactoperoxidase, was detected [1, 5, 6, 10, 13, 16, 26], no radiation-induced changes concerning the same proteins were reported in other studies [7, 9, 17].

Thus, the purpose of the present study was to compare the relative amount of low molecular weight salivary proteins in irradiated patients and healthy subjects using reverse-phase high-pressure liquid chromatography (HPLC) for separation of proteins.

Materials and methods

Ten patients aged between 45 and 71 years (mean 58.1 years) with malignant tumours localized in the lower two thirds of the craniofacial system participated in this study. Tumour therapy involved irradiation with a total dose of 60 Gy. The irradiation field included the parenchyma of all major salivary glands. None of the patients had undergone surgery of the salivary glands or was receiving any medication known to affect salivary gland function. In the irradiated patients suffering from hyposalivation, saliva was collected 6–12 months after irradiation. The patients were fully informed about the procedure, the aim of the

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study and gave their written informed consent. The control group involved ten healthy volunteers who were 34 (±10) years old and did not suffer from any salivary gland disease. In both groups, unstimulated saliva was collected on ice over a 5-min period, 2 h after breakfast between 10.00 and 11.00 a.m. Saliva was immediately centrifuged (4,000×*g*, 10 min), filtered (0.2 μm RC25, Sartorius) and frozen (−70°C).

Saliva samples (50 μl) were separated on a C8 (octyl acylated) Zorbax column with a pore size of 0.03 μm by reverse-phase liquid chromatography (RPLC, Hewlett Packard, LC 1100) and a water/acetonitrile (ACN) gradient containing 0.05% trifluoroacetic acid as ion-pairing reagent. The chromatograms were developed with a gradient between 5 and 95% ACN (Fig. 1). The total protein concentration was determined by addition of the peaks in the

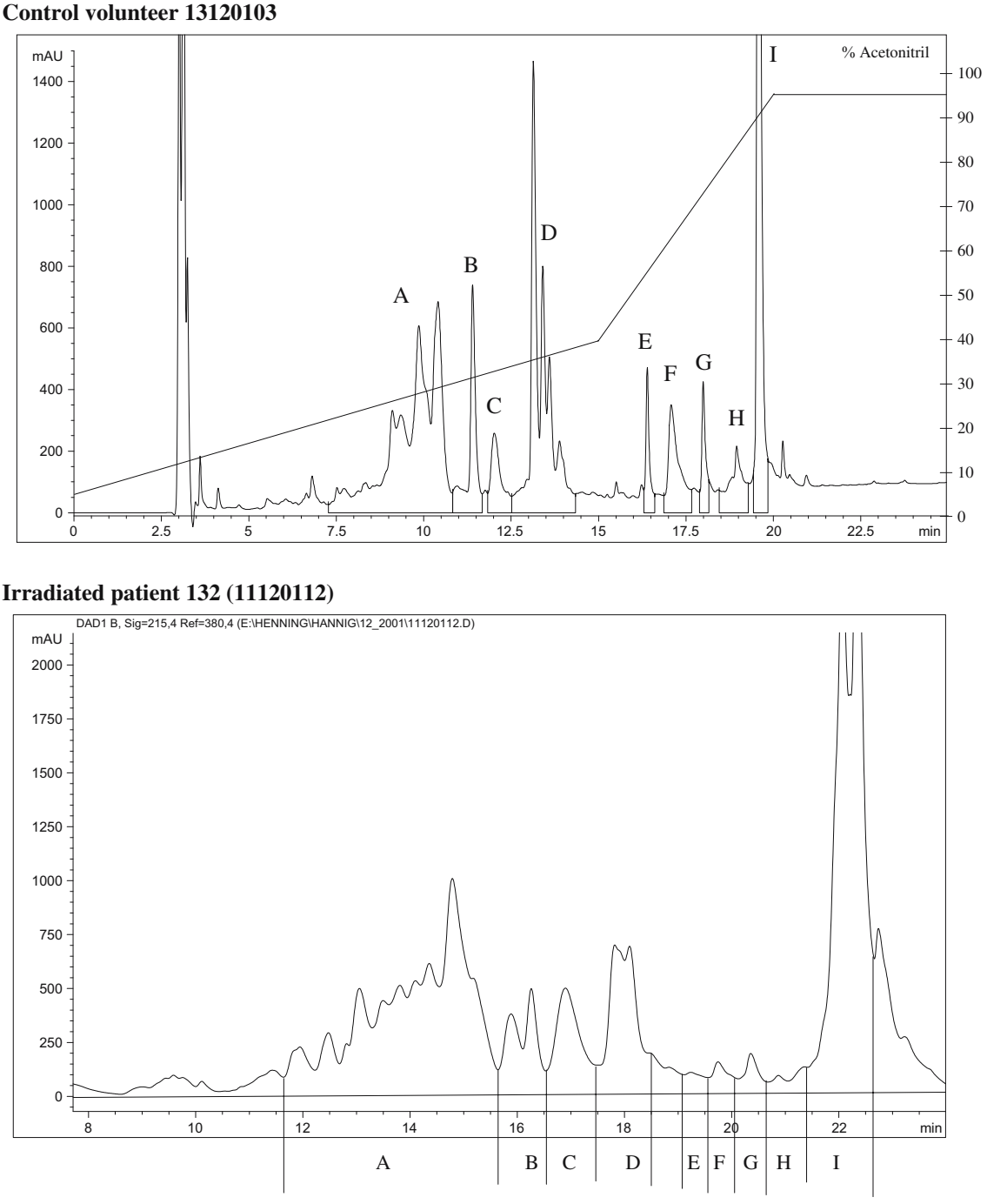

Fig. 1 Example of separation of saliva by reverse-phase liquid C8 chromatography with an acetonitrile gradient and at least nine components in the eluate (*top*, control volunteer; *bottom*, irradiated patient)

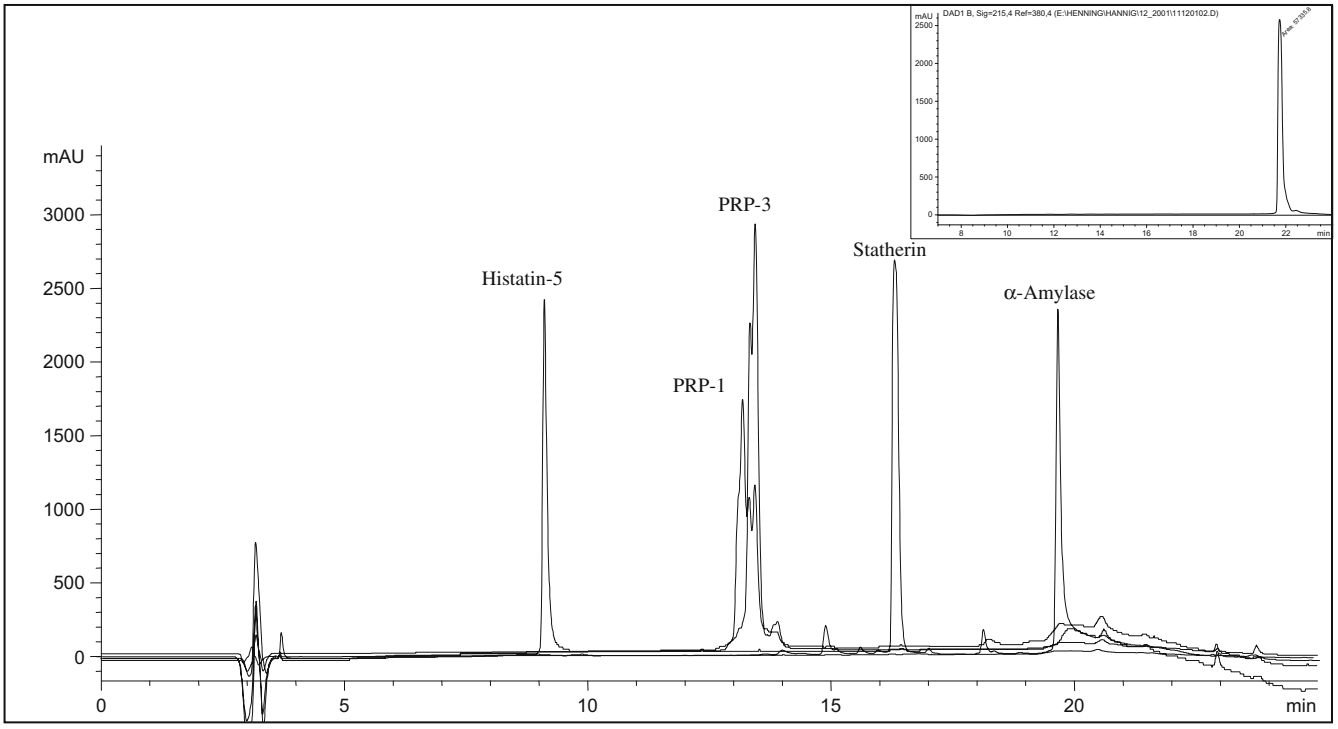

Fig. 2 Example of co-elution of reference proteins by reverse-phase liquid C8 chromatography. *Inset* shows the profile of bovine serum albumin 500 μg/ml for quantification purposes

RPLC recorded at a wavelength of 214 nm. Protein peaks were identified by co-elution of reference proteins (Fig. 2, histatin-5[1], PRP-1[2], PRP-3[2], statherin[2], α-amylase[3]) and by means of mass spectrometry with electrospray ionization (ESI-MS) in a range of mass/charge of 0–1,600 (HP MSD, G 1946A). The obtained mass spectra from multiple-charged ions were transformed into a calculated molecular weight using deconvolution programme provided by the MSD manufacturer. Close matches with the protein database [3] provided further confirmation of the identity of salivary proteins or peptides. In this way, saliva proteins were separated into individual peaks as statherin and α-amylase or partial resolved peaks as acidic or basic proline-rich proteins (PRPs) including histatins (Fig. 1). For quantification purpose, the partially resolved peaks were combined into groups, and the integrated peak areas (referred to as A–I) were related to the total area (100%), which was compared with a plot of 500 μg/ml bovine serum albumin. The latter was used as standard to calculate the amount of protein of the peaks. Repeated separations showed high reproducibility between 1 and 5% [14].

Using SPSS for windows (version 11.5), statistical analysis of the data was performed with the Student's *t* test and Mann–Whitney *U* tests, with the level of significance defined at $p \leq 0.05$.

Results

Mean salivary flow rates over the 5-min period of saliva collection were 84±56 μl in irradiated patients and 665±37 μl in the control group and differed significantly between both groups. However, the total protein concentration of saliva was quite similar in these groups, with 2,972±2,268 μg/ml in irradiated patients and 2,674±536 μg/ml in volunteers, and did not reveal significant differences. However, the high variation in irradiated patients points to the different convalescence of secretion after x-ray therapy. Typical chromatograms are shown in Fig. 1. Nine protein fractions (referred to as A–I) were identified in the saliva from irradiated patients as well as from the healthy volunteers (Table 1).

Peak A including histatins and bPRPs, peak D representing aPRPs and peak I representing α-amylase amount together to nearly 80% of total proteins in saliva of healthy volunteers. Since peak A of three irradiated patients was far above average while all other patients showed lower values, the mean between both groups did not differ significantly. However, the difference between aPRPs was statistically significant between the groups. Healthy volunteers demonstrated 456 μg/ml and exceeded twice the corresponding value of irradiated patients. The concentration of salivary amylase in both groups did not differ significantly (*t* test,

[1] Supply from FG Oppenheim (Boston, MA, USA).

[2] PRPs and statherin from N Stromberg (Umea, Sweden) were acknowledged.

[3] α-Amylase from Sigma was used.

Table 1 Nine protein peaks of RPLC separation of saliva (in micrograms of protein per millilitre) from xerostomic irradiated patients (n=10) or healthy control volunteers (n=10 each) and amount of saliva collected (in microlitres per minute)

Peaks		Irradiated patients (n=10)	Control volunteers (n=10)
A	Histatine, basic PRP	1,074±1,314	1,190±313
B	7.3, 7.6, 9.3 and 9.6 kDa	205±196	165±69
C	Unknown	249±298	114±107
D	Acidic PRP	250±218	456±139*
E	Statherin	81±89	36±18
F	5.8 kDa	62±59	88±30
G	Cystatin, 14.3 kDa	79±58	58±25
H	Cystatin, 14.2 kDa	271±252	91±46
I	α-Amylase	702±471	476±191
Total (μg/ml)		2,972±2,268	2,674±536
Saliva (μl/min)		420±278	3,323±185

RPLC Reverse-phase liquid chromatography, *PRP* proline-rich protein
*$p \leq 0.05$

p=0.052). Statherin, which could be well identified, amounted to 1.3–2.7% in both groups without considerable difference. The same was observed in cystatins with 2–3% for peak G and 3–9% for peak H. The chromatographic separation (Fig. 1) showed the qualitative similarity of salivary composition between irradiated patients and healthy volunteers, but a tendency to a lower protein concentration being statistically evident for fraction D could be seen.

Discussion

Six to twelve months after radiation therapy, patients suffered from severe xerostomia; they produced only 80 μl saliva/min, which was eight to ten times less than that in the control volunteers. The salivary protein concentration did not differ significantly between the groups and corresponds to values reported by Dodds et al. [8], with 2.72 mg/ml from an HPLC investigation. Centrifugation and deep-freeze storage of saliva might have caused a decrease in protein concentration; however, the general protein pattern of the salivary sample will not be changed due to this pretreatment [25]. The filtration of saliva with removal of large molecules as mucins which have high molecular weights of up to 40 million [20] was essential for the reproducibility of the RPLC analyses.

With the exception of statistically significant decrease in acidic PRPs (fraction D), no other differences were recorded concerning the salivary protein concentration in the irradiated patients compared with the control group. This finding is in accordance with previously published data on the unchanged salivary protein concentration after irradiation [2, 9]. Increased salivary protein concentration following irradiation was observed in other previous studies [7, 13, 16]. This is in accordance with results of particular individual irradiated patients in the present study. Such an increase could be the reason for the high variation of protein concentrations shown in Table 1. These results could be explained by different times of saliva collection after irradiation therapy or by different modes of sampling in various studies. In the present study, salivary protein composition was analysed on samples from saliva collected in patients with radiation-induced hyposalivation 6–12 months after cessation of the radiotherapy. Probably, this period caused different stages of regrowth of salivary acinar cells.

The RPLC analysis of these salivary samples indicated that the concentration of some salivary proteins was not altered due to radiation and related hyposalivation, whereas the concentration of other proteins decreased significantly (aPRPs) or in tendency (histatins, bPRPs). The apparently increased mean values (e.g. amylase, statherin, cystatins) were a consequence of high variations in irradiated patients compared with the control group.

Salivary amylase comprises up to 40–50% of total protein in parotid saliva. Samples of mixed saliva in the presented trial contained 17–23% of amylase. It serves as an indicator for the protein synthesis in the acinar cells and, thus, reflects the injury of the parotid gland during radiotherapy [1, 6, 7, 16]. It would be expected to find a decreased salivary concentration of amylase after irradiation. This was found in four patients only. Other authors did not find any significant changes in the amylase concentration of saliva due to irradiation [9, 17]. The latter findings are in accordance with the results of the present study indicating no significant alterations in the amount of salivary amylase.

Data concerning the radiation-induced changes on low molecular weight salivary proteins in irradiated patients are sparse. High concentrations of lactoferrin, lysozyme and salivary peroxidase were observed during radiotherapy, but most of the values return to pretreatment levels after cessation of the radiotherapy [16]. The present study reports for the first time findings on the concentration of statherin, histatin, cystatin and acidic PRPs in unstimulated saliva of irradiated patients. Significantly decreased salivary concentration of acidic PRPs was recorded, whereas cystatin and statherin remained unchanged between irradiated patients and control group. However the higher variation of cystatin and statherin pointed to a distinct influence of radiation on their production. These proteins are of physiological importance for the function of saliva. They have innate immunity properties concerning microbial adhesion or tooth tissue homeostasis, and they may provide diverse binding sites for bacterial lectin-like proteins [24]. The reported differences might be of significant influence concerning the protective properties of saliva in hyposalivated patients whose host-derived background might differently contribute to the defence against xerostomic alterations of the soft and hard dental tissues.

In summary, up to now, no extensive prevalence data were available for xerostomic-induced alterations in saliva composition since such analyses are not routinely performed [19]. Present RPLC data show that irradiation has a significant specific influence on protein composition of saliva; its acidic PRP concentration decreased, probably with biological loss of activity for maintaining oral health.

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