Eur Arch Otorhinolaryngol (2016) 273:4501–4506
DOI 10.1007/s00405-016-4159-2

HEAD AND NECK

The protective effects of different treatments on rat salivary glands after radiotherapy

Murat Konak[1] · Hakan Cincik[2] · Evren Erkul[3] · Zafer Kucukodaci[4] · Atila Gungor[3] · Sevim Ozdemir[5] · Engin Cekin[2] · Volkan Arisan[6] · Mesut Mutluoglu[7] · Murat Salihoglu[3]

Received: 28 April 2016 / Accepted: 15 June 2016 / Published online: 20 June 2016

Abstract This study was aimed to evaluate the efficacy of treatment modalities for minimizing salivary gland damage caused by radiotherapy. Forty rats were divided into five groups. Group 1 had no irradiation or any treatment. Group 2 underwent only 15 Gy single dose radiotherapy. *N*-acetylcysteine, dexamethasone, hyperbaric oxygen treatment were given, respectively to the group 3, 4 and 5 for 5 days. 15 Gy single dose radiotherapy was applied to the group 3, 4 and 5 on the second day. Pyknosis, lysis, and vacuolization were examined in ductal cells and pyknosis, lysis, vacuolization, inflammation and collective duct damage in acinar cells. Dexamethasone and hyperbaric oxygen did not prove to have a positive effect on acinar and ductal cell. *N*-acetylcysteine-applied group had statistically significantly lower amount of damage. We determined that the decrease of ductal and acinar cell damage in parotid glands of *N*-acetylcysteine-applied rats was more distinct and statistically.

Keywords Radiotherapy · Salivary glands · *N*-acetylcysteine · Hyperbaric oxygen · Corticosteroid

✉ Hakan Cincik
hakancincik1@yahoo.com

[1] Gulhane Military Medical Academy, Haydarpasa Training Hospital, Istanbul, Turkey

[2] Department of Otorhinolaryngology, Medipol University, Kosuyolu/Kadiköy, 34718 Istanbul, Turkey

[3] Department of Otorhinolaryngology, Gulhane Military Medical Academy, Haydarpasa Training Hospital, Istanbul, Turkey

[4] Department of Pathology, Gulhane Military Medical Academy, Haydarpasa Training Hospital, Istanbul, Turkey

[5] Department of Radiation Oncology, Istanbul University Cerrahpasa Medical Faculty, Istanbul, Turkey

[6] Department of Oral Implantology, Istanbul University Faculty of Dentistry, Istanbul, Turkey

[7] Department of Underwater and Hyperbaric Medicine, GATA Haydarpasa Training Hospital, Istanbul, Turkey

Introduction

Radiotherapy (RT) is a treatment modality that is used either as the sole means or in combination with surgery and chemotherapy. It is used as part of the treatment process in 50–60 % of all cancer patients [1]. The aim of RT is to apply a high enough radiation dose to the tumor while sparing the healthy tissues. One of the most noticeable side effects of ionized radiotherapy application on the head and neck region is xerostomia due to salivary gland damage. Xerostomia may cause deglutition and speech disorders, impairment in a sense of taste, dental cavities and oral infections over time; therefore, reducing the quality of life of patients [1–3].

Ionizing radiation damages the DNA and leads the cell into apoptosis. This fatal damage to the DNA may either could be incurred by direct ionizing effect of radiation, or indirect ionization; forming free oxygen radicals (FOR) as a result of the ionization of water [4]. Treatment methods such as hyperbaric oxygen (HBO) and corticosteroid therapy are applied to protect healthy tissues from the harmful effects of RT [1, 5–8].

In our study, we evaluated the efficacy of *N*-acetylcysteine (N-AC), hyperbaric oxygen and dexamethasone (DEX) treatment in preventing salivary gland damage resulting from RT in rats.

Materials and method

After obtaining Marmara University (Istanbul/Turkey) Animal Research Ethical Committee approval (32.2010.mar/09.04.2010), 40 healthy Wistar albino (*rattus rattus norvegicus*) male rats, aged 2 months and weighing 275 g (250–300) were divided randomly into five groups of eight at the beginning of the study. International standards regarding the animal care and handling have been followed during the experiments.

Study protocol

Group 1 (negative control group): They were kept in the same place and had no treatment.

Group 2 (positive control group): 15 Gy single dose RT was applied on the head and neck area on the second day of the study.

Group 3 (RT + N-AC): 1 g/kg/day *N*-acetylcysteine (N-AC) (Asist 300 mg/3 mL ampoule, Bilim Pharmaceuticals, Turkey) was injected intraperitoneally (IP) for 5 days. 1 h after the N-AC application on the second day, 15 Gy single dose RT, was applied on the head and neck area.

Group 4 (RT + DEX): 1 mg/kg/day dexamethasone (DEX) (Dekort 8 mg/2 mL ampoule, Deva Pharmaceuticals, Turkey) was injected IP for 5 days. 1 h after the DEX application on the second day, 15 Gy single dose RT, was applied on the head and neck area.

Group 5 (RT + HBO): This group was exposed to hyperbaric oxygen treatment. 2,4 ATA of hyperbaric oxygen 90 min daily for 5 days. 1 h after the second session of HBO on the second day of the experiment, 15 Gy single dose RT, was applied on the head and neck area. HBO was performed in a specially designed, monoplace hyperbaric oxygen therapy unit. Eight rats were accomodated in the hyperbaric chamber in each HBO session. 2,4 ATA of pressure was obtained in 5 min with 100 % oxygen. Each therapy session consisted of 90 with 5 min of compression, 5 min of decompression and 80 min of treatment.

Radiotherapy application

A total of 32 rats in groups 2, 3, 4, 5 were put under deep anaesthesia for 30–45 min with the help of intraperitoneal 100 mg/kg ketamine HCI (Ketalar® 50 mg flk, Pfizer, USA) on the second day of the experiment. To irradiate the rats at a time, lead blocks defined the irradiation field which was 30 cm in length with a 4 cm of field margin, and shielded other parts. A dose of 15 Gy radiotherapy was applied on the head and neck region. Teletherapy device [Cirus model (CGR CisBio)—serial no: 90116, Co-60 source, France] was used for RT.

Histopathological examination

General anaesthesia was induced with intraperitoneal 100 mg/kg Ketamine Hydrocloride (Ketalar® Flakon, Pfizer, USA) on the seventh day of study, and parotid and submandibular salivary glands were excised.

Sections from parotid and submandibular glands had been fixed in a 10 % buffered formalin solution for 24 h and then sent through standard paraffin-embedding. Sections were stained with haematoxylin–eosin (HE) after the routine procedures. To reveal the cellular damage caused by ionized radiation, pyknosis, lysis, and vacuolization were evaluated in ductal cells. Pyknosis, lysis, vacuolization, inflammation and collective duct damage were evaluated as well in acinar cells. HE stained sections were scored at 400× magnification under Nikon E-600 light microscope (Table 1). Pyknosis, lysis and vacuolization scoring were made according to their presence in the cells. Presence in 1–5 % of the cells were scored as + (mild), in 5–10 % of the cells as ++ (moderate), in 10–25 % of the cells as +++ (severe), and in >25 % of the cells as ++++ (very severe). Inflammation and collective duct damage were scored according to the level of the pathology.

Statistical evaluation

Descriptive statistics were used to determine the average, median, minimum, maximum and 95 % confidence interval values of the scores obtained. Normality of the data distribution was examined with D'agostino Pearson Omnibus normality test. The significance of the differences between groups was primarily evaluated with Kruskall Wallis test. Then Dunn's multiple comparison test was used for pairwise comparisons (post hoc test) in groups which had statistically significant differences. Probability (p) levels below 0.05 were considered statistically significant. All analyses were carried out using GraphPad Prism 5.0 (San Diego, California, ABD) program.

Results

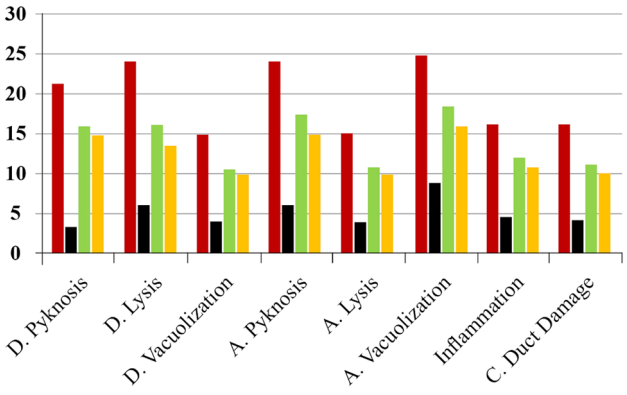
All of the rats completed the study uneventfully. Parotid gland was more remarkably affected compared with submandibular gland in terms of ductal and acinar cell damage ($p < 0.05$). Therefore, data derived from the parotid gland was taken into consideration while evaluating the statistical results of the effects of radiotherapy comparing treatment modalities.

Table 1 Descriptive statistics—*p* values obtained by comparing the control group (group 2) with study groups (group 3, 4, 5)

	Group 3 (RT + N-AC)	Group 4 (RT + DEX)	Group 5 (RT + HBO)
Ductal pyknosis	<0.005	>0.05	>0.05
Ductal lysis	<0.005	>0.05	>0.05
Ductal vacuolization	<0.001	>0.05	>0.05
Acinar pyknosis	<0.005	>0.05	>0.05
Acinar lysis	<0.001	>0.05	>0.05
Acinar vacuolization	<0.005	>0.05	>0.05
Inflammation	<0.001	>0.05	>0.05
Collective duct damage	<0.001	>0.05	>0.05

HBO hyperbaric oxygen, *RT* radiotherapy, *N-AC N*-acetylcysteine, *DEX* dexamethasone

Table 2 Comparison of average values of control and treatment groups (*Y* axis is the average scores of histopathological examinations in all groups, *X* axis; red bar: group 2, black bar: group 3, green bar: group 4, yellow bar: group 5)



HBO hyperbaric oxygen, *RT* radiotherapy, *N-AC* N-acetylcysteine, *DEX* dexamethasone, *D* ductal, *A* acinar, *C* collective

With regard to parotid gland; when comparing treatment groups with positive control group (group 2), N-AC group (group 3) was determined to have statistically significantly low ($p < 0.005$) damage in terms of ductal pyknosis, ductal lysis, acinar pyknosis and acinar vacuolization. Statistically significantly low damage on a more distinct level ($p < 0.005$) was found in terms of ductal vacuolization, acinar lysis, inflammation and collective duct damage (Table 1). Average values of histopathological examinations were showed in Table 2.

With regard to parotid gland, no statistically significant difference ($p < 0.05$) was found between positive control group and group 4 (RT + DEX) and group 5 (RT + HBO) in terms of all parameters (Fig. 1).

Discussion

Xerostomia is a clinical symptom resulting from reduced saliva secretion [9]. It has a well known course and is a frequently observed side effect occurring after the application of ionizing radiation on the head and neck [1]. Huguenin et al. evaluated cases with head and neck cancer in terms of life quality on the fifth year after radiotherapy and divided the patients into three groups as glottic, nasopharynx and other head-neck cancers. They reported that xerostomia was the most frequent side-effect in all groups [3].

In a study, histopathological examination was performed on salivary glands of rats at 30th min, 1st, 4th and 24th h after the application of 15 Gy single dose radiotherapy on the head and neck region. Histopathological changes such as acute inflammation, cellular lysis and focal acinar loss were reported to occur following the first 24 h after radiation [10]. Taking account of this study, we also applied 15 Gy single dose radiotherapy to create xerostomia. To confirm that this amount of dose causes salivary gland damage, we formed one negative and one positive control group and compared them. We demonstrated the effects of RT on salivary glands of rats and determined the parameters according to these findings.

The fact that free oxygen radicals play an important part in the development of side-effects (mucositis, xerostomia, etc.) following RT on the head and neck region which is accepted by researchers [1, 4]. There are publications that indicate the beneficial effect of N-AC, which is the precursor of intracellular cysteine and glutathione, in diseases related with oxidative stress [11–13]. It was pointed out the effectiveness of N-AC in reducing the DNA damage that is increased by UV-A, UV-B and visible radiation [11, 12]. There are also various publications indicating that the treatment modalities such as hyperbaric oxygen, N-AC and DEX, reduce the oxidative damage as we used in our study [5, 7, 13, 14].

Lipid peroxidation caused by radiotherapy and other reasons, increases the cell membrane permeability and oxidation of proteins located on the cell membrane, thus largely impairing functioning of the cell membrane [1, 15]. There is strong evidence that N-AC can maintain the integrity of the cells by inhibiting lipid peroxidation and protein oxidation [11, 13]. In a study carried out with old

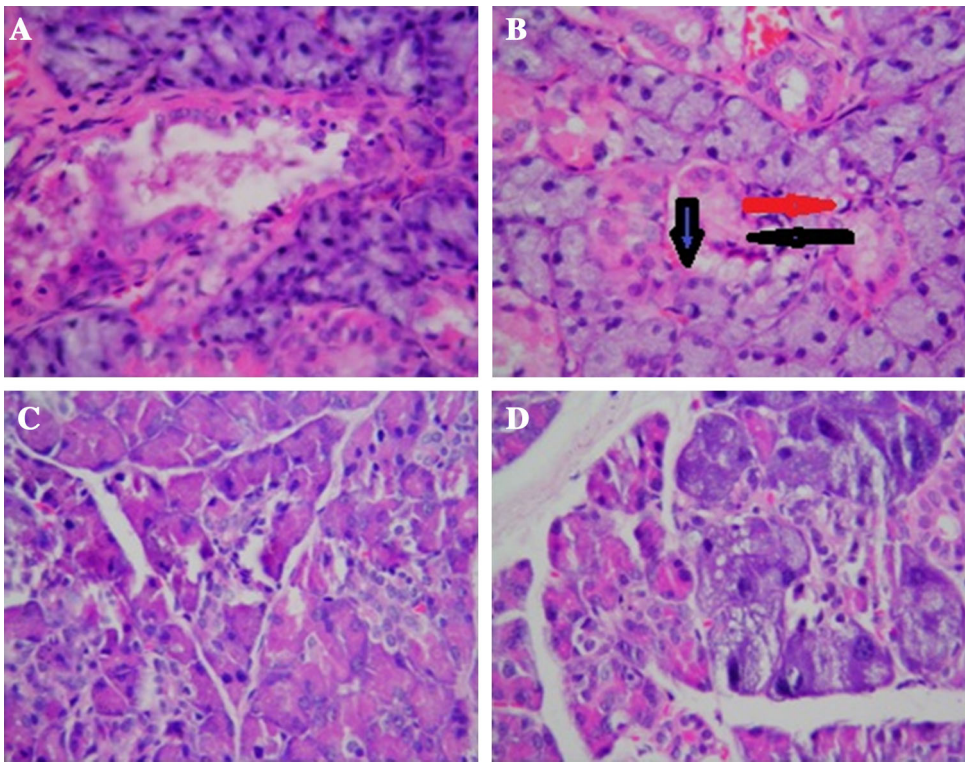


Fig. 1 **a** Ductal lysis seen after dexamethasone treatment (HE ×400). **b** Pyknosis (*black arrow*), vacuolization (*blue arrow*) in intralobular cells and hyperchromatization in acinar cells (*red arrow*) observed after hyperbaric oxygen treatment (HE ×400). **c** Acinar cell lysis in parotid gland of radiotherapy-applied rat (HE ×100). **d** Vacuolization in radiotherapy-applied parotid gland (HE ×100)

mice, lipid peroxidase and protein carbonyl activity in mitochondrias of N-AC-applied group were determined to be lower [16]. In addition to these, it should be emphasized that NAS is effective in preventing DNA fragments resulting from radiation and preventing cancer development. Reliene et al. demonstrated that N-AC-supplemented diet reduced biological indicators of carcinogenesis such as DNA damage in mice that had mutation in ATM gene [17].

Neal and colleagues applied 18 Gy single dose ionizing radiation on mice and reported that isomers of N-AC, though limited, had radioprotective efficiency on lung, liver, spleen and red blood cells [13]. In our study, protective effects of N-AC against radiotherapy-induced salivary gland damage were evaluated. Our findings bear resemblance to those of Neal.

Nevertheless, He et al. [18] compared the protective effects of N-AC and ascorbic acid against lipid peroxidation resulting from UV-B and double-strand breaks. As a result, they found that ascorbic acid was significantly more protective.

Mansour et al. [11] applied single dose whole body radiation of 6 Gy on rats and evaluated radiation-induced hepatotoxicity. Differently from our study, 1 g/kg/day of N-AC was administered intraperitoneally for 7 days before the radiotherapy and radioprotective efficiency was investigated in prophylactic terms. Rats were sacrificed 24 h after radiotherapy. Biochemical and histopathological examination results showed an increase in antioxidant enzyme levels and decrease in DNA damage. In our study, single dose radiotherapy was applied at 15 Gy instead of 6. On the second day, 1 h after the administration of the second dose of N-AC intraperitoneally, radiotherapy was applied on the head and neck region of the rats. Thus, we could evaluate not only the prophylactic, but also the therapeutical efficiency of *N*-acetylcysteine. The results we obtained support those of Mansour in terms of antioxidant efficiency.

Hyperbaric oxygen treatment is thought to reduce chronical tissue hypoxia and progressive microvascular loss in physiopathological process resulting from radiation. Many researchers agree that subsequent HBO treatments increase formation of connective tissue, capillaries and epithelium [19] and help wound healing [20]. Dizdar et al. [6] applied 20 Gy on the head and neck region of rats in five fractions in their study. Subsequent to radiation, treatment group was applied 2,6 ATA of hyperbaric oxygen 2 h daily for 30 days. Rats were sacrificed on the 60th, 75th, 90th days, and histopathological examination was performed on their salivary glands. They reported that HBO treatment can reduce radiotherapy-induced damage. While Dizdar et al. evaluated late-term tissue damage; we studied the efficiency in reducing early stage tissue damage. However, we could not establish that it reduced early stage parotid gland damage resulting from radiation.

While corticosteroids are reported to have positive effects on inhibiting tissue damage due to radiation

[21, 22], Berdjis [23] and Cladwell [24] reported that steroids even in small doses can cause side effects subsequent to radiation on renal region. In a study about the radiation damage on rectums of the dogs which was published by Stryker et al. [25], prednisolone was reported to have no positive effect at all on preventing early term complications due to radiation, and it was further emphasized that it actually aggravates late-term complications. We used dexamethasone in our study and did not find any protective efficiency with regard to reducing RT-induced parotid gland damage in rats.

Wang et al. [26] shows that during RT the parotid glands shrink more than the submandibular glands and parotid gland is more radiosensitive because the parotid gland is more serous and the loss of serous acini has grater loss than mucous acini after irradiation. However, they concluded that mechanism of this loss is complex and not certain because there are some several studies that show no significant difference between two glands [27, 28]. In our study, the parotid gland was more remarkably affected compared with submandibular gland in terms of ductal and acinar cell damage.

Conclusion

We determined that the decrease of ductal and acinar cell damage in parotid glands of *N*-acetylcysteine-applied rats was more distinct and statistically significant than those of all other treatment groups, and besides it had a protective effect on their parotid glands. Additionally we found that HBO-applied group had better protective efficiency than dexamethasone group; however, neither of each result was statistically significant. However, clinical studies are needed for evaluating its positive effects on humans.

Compliance with ethical standards

Marmara University (Istanbul/Turkey) Animal Research Ethical Committee approval (32.2010.mar/09.04.2010). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest All authors disclose no conflicts of interest.

References

1. Cox JD, Ang KK (2003) Part 3 head and neck. In: Cox JD, Ang KK (eds) Radiation oncology: rational, technique, results, 8th edn. Mosby, Missouri, pp 145–310
2. Marmiroli L, Salvi G, Caiazza A, Di Rienzo L, Massaccesi M, Murino P, Macchia G (2005) Dose and volume impact on radiation-induced xerostomia. Rays 30(2):145–148
3. Huguenin PU, Taussky D, Moe K, Meister A, Baumert B, Lütolf UM, Glanzmann C (1999) Quality of life in patients cured from a carcinoma of the head and neck by radiotherapy: the importance of the target volume. Int J Radiat Oncol Biol Phys 45:47–52
4. Hall EJ (2000) Radiobiology for the radiologist, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 47–85
5. Pasquier D, Schmutz J, Lartigau E (2006) Radio induced lesion in normal tissues. In: Mathieu D (ed) Handbook on hyperbaric medicine. Springer, Dordrecht, pp 363–401
6. Dizdar Y, Olgaç V, Aktaş Ş, Ağaoğlu FY (2003) Hiperbarik Oksijen Uygulamasının Radyasyona Bağlı Parotis Hasarına Etkileri. Türk Onkol Derg 18(1):6–11
7. Owoeye O, Malomo AO, Elumelu TN, Salami AA, Osuagwu FC, Akinlolu AA (2008) Radiation nephritis: anti inflammatory effect of dexamethasone in adult Wistar rats (*Rattus novergicus*). Int J Morphol 26(1):69–74
8. Vissink A, Burlage FR, Spijkervet FK, Jansma J, Coppes RP (2003) Prevention and treatment of the consequences of head and neck radiotherapy. Crit Rev Oral Biol Med 14(3):213–225
9. Guggenheimer J, Moore PA (2003) Xerostomia: etiology, recognition and treatment. J Am Dent Assoc 134:61–69
10. Nagler RM, Baum BJ, Fox PC (1993) Acute effects of X irradiation on the function of rat salivary glands. Radiat Res 136(1):42–47
11. Mansour HH, Hafez HF, Fahmy NM, Hanafi N (2008) Protective effect of *N*-acetylcysteine against radiation induced DNA damage and hepatic toxicity in rats. Biochem Pharmacol 75(3):773–780
12. Morley N, Curnow A, Salter L, Campbell S, Gould D (2003) *N*-acetyl-L-cysteine prevents DNA damage induced by UV-A, UV-B and visible radiation in human fibroblasts. J Photochem Photobiol B 72:55–60
13. Neal R, Matthews RH, Lutz P, Ercal N (2003) Antioxidant role of *N*-acetyl cysteine isomers following high dose irradiation. Free Radic Biol Med 34:689–695
14. Spapen H, Zhang H, Demanet C, Vleminckx W, Vincent JL, Huyghens L (1998) Does *N*-acetyl-L-cysteine influence cytokine response during early human septic shock? Chest 113:1616–1624
15. Sheldon JM, Zelefsky MJ (1998) Hypopharynx. In: Leibel SA, Phillips TL (eds) Textbook of radiation oncology. W. B. Saunders Company, Philedelphia, pp 477–496
16. Martínez M, Hernández AI, Martínez N (2000) *N*-acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria. Brain Res 855:100–106
17. Reliene R, Fischer E, Schiestl RH (2004) Effect of *N*-acetyl cysteine on oxidative DNA damage and the frequency of DNA deletions in atm-deficient mice. Cancer Res 64:5148–5153
18. He YY, Häder DP (2002) UV-B induced formation of reactive oxygen species and oxidative damage of the cyanobacterium *Anabaena* sp.: protective effects of ascorbic acid and *N*-acetyl-L-cysteine. J Photochem Photobiol B 66:115–124
19. Mayer R, Hamilton-Farrell MR, van der Kleij AJ, Schmutz J, Granström G, Sicko Z (2005) Hyperbaric oxygen and radiotherapy. Strahlenther Onkol 181:113–123
20. Gill AL, Bell CN (2004) Hyperbaric oxygen: its uses, mechanisms of action and outcomes. Assoc Phys QJM 97:385–395
21. Shaw PJ, Bates D (1984) Conservative treatment of delayed cerebral radiation necrosis. J Neurol Neurosurg Psychiatry 47:1338–1341
22. Ward HE, Kemsley L, Davies L, Holecek M, Berend N (1993) The effect of steroids on radiation-induced lung disease in the rat. Radiat Res 136:22–28
23. Berdjis CC (1960) Cortisone and radiation. III. Histopathology of the effect of cortisone on the irradiated rat kidney. Arch Pathol 69:431–439
24. Cladwell W (1971) The effect of prednisolone on fatal postirradiation nephritis in rabbits. Radiology 98:431–433
25. Stryker JA, Abt AB, Allegra JC, Mortel R, Uhlin S, Hepner GW (1976) The effect of prednisone and irradiation on the rectum in dogs. Radiology 121:183–187

26. Wang ZH, Yan C, Zhang ZY et al (2009) Radiation-induced volume changes in parotid and submandibular glands in patients with head and neck cancer receiving postoperative radiotherapy: a longitudinal study. Laryngoscope 119:1966–1974
27. Coppes RP, Vissink A, Zeilstra RJ et al (1997) Muscarinic receptor stimulation increases tolerance of rat salivary gland function to radiation damage. Int J Radiat Biol 72:615–625
28. Coppes RP, Vissink A, Konnigs AW (2002) Comparison of radiosensitivity of rat parotid and submandibular glands after different radiation schedules. Radiother Oncol 63:321–328