Acta Scientific Dental Sciences (ISSN: 2581-4893)

Volume 8 Issue 2 February 2024

Research Article

Early Evaluation of Minor Serous Gland Injuries Following Fractionated Radiotherapy for Head and Neck Cancer (Immunohistochemical Study)

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DOI: 10.31080/ASDS.2024.08.1784

Received: January 08, 2024

Published: January 22, 2024

Abstract

Objectives: This study aimed to analyse the histopathological and immunohistochemical expression of proliferating cell nuclear antigen (PCNA) and cytokeratin 17 in serous acini of Von Ebner glands of the irradiated versus non irradiated rats for head and neck malignancy to provide a more detailed profile of the radiation injury to the head and neck region.

Methods: Twenty male adult albino rats were divided into two groups; control group received no radiation and irradiated group received a radiation dose of 5 Gy daily for five consecutive days with a total dose of 25 Gy using therapeutic X-ray beam. One month after the last dose of irradiation, rats were euthanized and the tongue was carefully dissected out, fixed, and stained with H&E for routine histopathological examinations. Immunohistochemical staining was performed using antibodies specific for the expression of both proliferating cell nuclear antigen (PCNA) and cytokeratin 17 (CK17).

Results: Histological findings confirmed that the parenchyma of Von Ebner's gland in the radiation-exposed group had glandular atrophy characterized by loss of gland architecture, degenerated acini, and dilatation of the ductal system. Furthermore, there is a predominance of the fibrous component with the presence of fatty degeneration within the glandular tissue. Expression of PCNA in control Von Ebner gland revealed negative to weak reactivity, whereas the irradiated group showed moderate to strong staining in the gland parenchyma. Expression of CK17 in irradiated Von Ebner glands revealed significant moderate to strong reactivity in the acinar and ductal cells compared with weak to mild reactivity in control. The staining expression of irradiated group was varied from diffuse to locally concentrated at the apical cell part.

Conclusion: The severity and prevalence of PCNA and CK17 in the irradiated group from our results suggest a pathological effect that interferes with saliva production and/or secretion leading to xerostomia. Also, the intensity of PCNA expression in that group could be a pathogenic factor in carcinogenesis rather than the healing process in the serous apocrine glands.

Keywords: Radiotherapy; Cytokeratin 17; PCNA; Fractionated; Von Ebner Gland

Introduction

Salivary glands are a group of major and minor exocrine glands that drain saliva into the oral cavity. Minor salivary glands are distributed throughout most parts of the oral cavity, and their secretions directly engulf the oral tissues to maintain the healthy condition of the oral mucous membranes and teeth [1]. Saliva plays several key functions in the oral cavity including buffering, lubrication, antimicrobial, digestion, hormone regulation, and sense of taste [2]. Salivary gland insufficiency is a major problem that leads to severe adverse health outcomes including tooth decay, swallowing difficulties, taste disturbance and increased risk of periodontal disease resulting in diminished quality of life for these patients [3-6].

Head and neck cancer is a broad term that accounts for 5% of malignancies throughout the body, including that arise from the oral cavity, pharynx, larynx, and paranasal sinuses [7]. Radiation therapy for head and neck cancer often leads to unavoidable side effect to the healthy orofacial structures. Both major and minor salivary glands are sensitive to radiation, with early effects occurring within a few days of irradiation due to the high potential for parenchymal damage [8,9]. Side effects resulting from therapeutic radiation can be divided into early observed during or shortly after radiation, affecting salivary secretion, taste, and oral mucosa in general. Then come the long-term side effects that appear months or years after radiation, affecting teeth, bones, and muscles [10] in addition to delayed tooth eruption and are also considered carcinogenic [11].

Immunohistochemistry is a technique to detect an intracellular component that acts as an antigen by antigen-antibody reaction [12]. IgG is a common antibody used in immunohisto-chemistry and is produced by immunizing an animal with a specific antigen which mounts a humeral immune response to that specific antigen and produces an anti-epitope called a monoclonal antibody that can be isolated from the animal for use in the expression of that antigen intracellular [13]. Proliferating cell nuclear antigen (PCNA) is a nuclear protein that act as a cofactor for DNA polymerase-delta and plays a role in the initiation of cell proliferation [14]. Burgess ., *et al*., examined atrophy and regeneration of rat parotid glands, using immunohistochemistry proliferating cell nuclear antigen (PCNA) and observed many positive cells [15]. Cytokeratin intermediate filaments are a family of proteins encoded by different genes and expressed in various epithelial cells including salivary gland. Cytokeratin immunoreactivity constitutes an important biomarker because it is highly antigenic, stable and shows great fidelity of expression [16]. Our study aims to examine the immunohistochemistry of the adverse effect of fractionated radiotherapy of head and neck region on the intracellular structure of Von Ebner gland.

Materials and Methods

Twenty male albino rats weighing 160-180 gm ware included in this study. Rats were maintained in an animal health care facility under the supervision of the Ethical Committee of Laboratory Animal Colony, Faculty of Veterinary Medicine, Cairo University, Egypt. Animals were kept in polycarbonate cages (4 rats per cage) under 8-16 dark/light cycles and were given a mixture of hard and soft foods with unlimited access to water. The animals were divided into two equal groups: Group (1) represented control rats, and Group (2) (experimental group) received radiotherapy of 5 Gy/day for five consecutive days for a total of 25 Gy. At every day of radiation exposure, rats of both groups were restrained carefully and anesthetized by intraperitoneal injection of Thiopental sodium (E. I. P. I. Co 10th of Ramadan Egypt) 30 mg /kg body weight

At the day of radiation, the rats of the irradiated group were covered with a 5 mm thick lead sheet to protect vital organs, except for the neck area that will be exposed to the radiation tube. Rats received radiotherapy between 8:00-12:00 noon at a dose of 5 Gy (1 Gy/min for 5 min) daily for five days using a therapeutic X-ray beam [Philips SL 75.5] operating at 235 kV, 15 mA. The maximum field size is 40 x 40 mm at 43 cm from focus.

One months after the last dose of irradiation, rats from both groups were euthanized by intracardiac injection of pentobarbital, and the tongue was carefully dissected out and fixed in 10% neutral buffered formalin for ten days. Sections were made using 5-6 µm thick paraffin-embedded tissue and stained with hematoxylin and eosin (H and E) for histo-pathological examinations.

Immunohistochemical staining was performed using antibodies specific for the expression of both PCNA and CK17. Sections were mounted on a silicone-coated glass slide, deparaffinized in xylene, and rehydrated with a descending series of ethanol. After blocking endogenous peroxidase activity with methanol containing 0.3% H2O2 for 30 minutes, antigen was retrieved by microwave heating for 5 minutes, and then the blocking reagent was applied for 10 minutes to reduce nonspecific staining.

Primary monoclonal antibody (Dako) against PCNA was applied using the Streptavidin biotin-peroxide complex (SABC) method and methyl green pylonin counter stain. Immunoreactivity of PCNA was considered negative if less than or equal to 10% (average 5%) of the cells stained positive with intense homogenous brown. The intensity of the PCNA expression was graded as weak 11-25% (average 18%), mild 26-50% (average 38%), moderate 51-75% (average 63%), and strong 76-100% (average 88%).

Cytokeratin 17 was identified by monoclonal anti-CK17 E3 antibody (Sigma) using labelled streptavidin biotin (LSAB) method and Hematoxylin counter stain. The positive staining reaction appeared in the form of brownish staining which reflect the intracellular distribution of CK17 within the tissue compartments. The intensity of the stain was assessed semi quantitively and scored as follow: negative (0), weak (1), mild (2), moderate (3), or strong (4) staining.

Data analysis was performed using SPSS, version 23 (IBM Inc., Chicago, Illinois, USA). Quantitative data were written as mean ± standard deviation (SD) and ranges when their distribution was found to be parametric by normality test. Comparison between groups with quantitative data and parametric distribution was performed using an independent T-test. The confidence interval was set to 95% and the acceptable margin of error was set to 5%. Therefore, the P value was considered significant at ≤ 0.05.

Results

Some cases of death occurred in the first few hours of exposure to radiation, and this may be due to complications from anesthesia, and they were excluded from the experiment and replaced with other animals. All irradiated and control rats that survived were in a good health until sacrification.

Von Ebner gland of control group revealed a parenchymal tissue filled with closely packed serous acini and duct system. Serous acini are spherical and composed of narrow central lumen encircled by pyramidal shape serious cells with basophilic cytoplasm and round basally situated nuclei. The lobules contain both intercalated, striated ducts and with some instance interlobular excretory ducts. These parenchymal elements are supported by connective tissue stroma that divides the gland into lobes and lobules (Figure 1).

Von Ebner's gland of the irradiated group revealed atrophic changes characterized by loss of gland structure and decreased

acinar size with delimitation of the lumen. This atrophic change was accompanied by an increase in the amount of fibrous stroma,

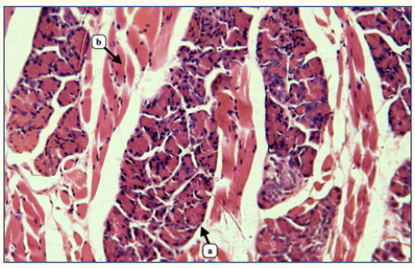

Figure 1: Von Ebner's gland of control group showing closely packed serous acini in basally situated nuclei (a), bundles of tongue muscle (B), (H&E x200).

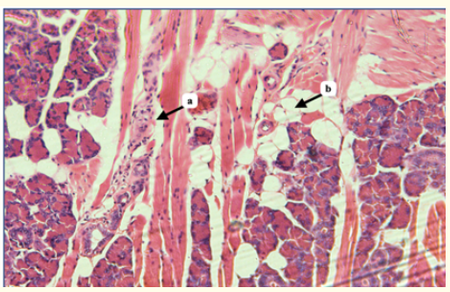

Figure 2: Von Ebner glands of irradiated group showing Degenerated serous acini with loss of gland architecture (a), fatty spaces among the serous acini (b), (H&E.x100).

which in some samples may reach complete fibrosis and the spread of adipose tissue in other samples. Some of the irradiated gland acini were enlarged, swollen, and demonstrated a proliferating activity in the form of increased mitotic figures, many of them are in abnormal value. The duct system appeared dilated while their cells became flat instead of cubical in normal glands. The blood vessels were dilated with extravasations of blood within the glandular tissue (Figure 2).

PCNA immunostaining

Immunoreactivity of PCNA in Von Ebner's gland in most of the control rats revealed negative expression for both acinar and ductal cells (Figure 3). In some animals, acinar cells showed positive expression ranging from weak to moderate intensity (tab.1). PCNA expression in most of the irradiated Von Ebner's gland revealed significant moderate to strong staining in the gland parenchyma compared with the control group (Figure 4). Some specimens of irradiated group showed acinar expression varied from weak to mild, average was summarized in (Table 1). The mean value of PCNA expression following on month after stopping radiation showed significant increase than that of the control group (mean ± SD = 12.2 ± 10.942) as the P-value was < 0.05 (Table 2,3).

CK17 immunostaining

The immunoreactivity of anti-cytokeratin E3 antibody for de-

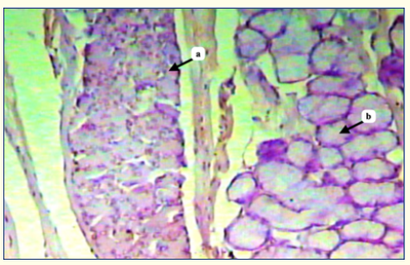

Figure 3: PCNA expression of normal Von Ebner and Weber glands showing weak reactivity of serous acinar nuclei (a) and negative reactivity in mucous acinar nuclei (b), (x200).

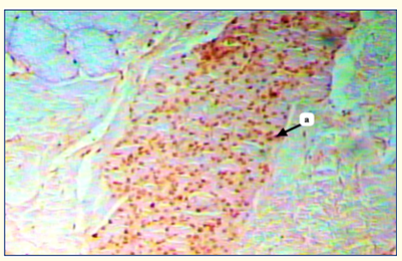

Figure 4: PCNA expression of radiated Von Ebner gland showing strong reactivity of serous acinar nuclei (a), (x200).

tection of CK17 using immunoperoxidase technique in control rat von Ebner glands revealed traces (week) to mild expression in many serous acini (Table 1). The staining pattern was either diffuse or concentrated in the basal cell portion with less staining in the apical portion of the acinar cells (Figure 5). Expression of CK17 in Von Ebner's glands of irradiated group revealed a distinct staining in both acinar and ductal cells. The staining of irradiated glands was of moderate to strong reactivity and varying from diffuse to locally concentrated at the apical cell part in contrast with control (Figure 6). The mean value of CK17 expression following on month after stopping radiation (mean ± SD = 2.00 ± 0.782) showed significant increase than that of the control group (mean ± SD = 1.025 ± 0.362) as the P-value was < 0.05 (Table 2,4).

Discussion

Damage to salivary glands is one of the most important harms of fractionated radiotherapy to head and neck area, which leads to deficiency in the production and secretion of saliva. Many researchers have stated that there is a great deal of uncertainty regarding the mechanisms of radiation-induced damage to the salivary glands [17,18].

Our results suggest that serous acinar cells respond to apoptosis in the form of atrophic changes following radiotherapy and

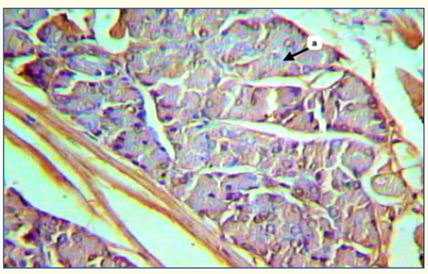

Figure 5: CK17 expression of serous acini of Von Ebner gland showing negative (a) to weak reactivity (b) in control group, and moderate (c) to strong reactivity (d) in radiated group, (x200).

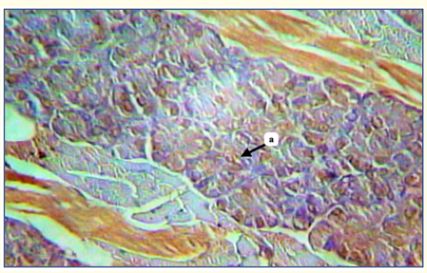

Figure 6: CK17 expression of radiated Von Ebner gland showing moderate to strong reactivity of serous acinar cells (a), (x200).

Average of PCNA and CK17 expression between groups				
	PCNA immunostaining		CK17 immunostaining	
Rats	Control group	Radiated group	Control group	Radiated group
1	5%	5%	0.50	0.75
2	5%	18%	0.75	1
3	5%	18%	0.75	1.5
4	5%	18%	0.75	2.00
5	5%	38%	1.00	2.00
6	5%	63%	1.00	2.00
7	18%	63%	1.25	2.25
8	18%	88%	1.25	2.25
9	18%	88%	1.25	3.00
10	38%	88%	1.75	3.25

Table 1: Average of PCNA and CK17 between groups.

Group Statistics of PCNA and CK17 expression					
	Group	N	Mean	Std. Deviation	Std. Error Mean
PCNA	1	10	12.2000	10.94227	3.46025
	2	10	51.2000	33.20241	10.49952
CK17	1	10	1.0250	.36228	.11456
	2	10	2.0000	.78174	.24721

Table 2: Group Statistics of PCNA and CK17 expression of both groups.

altered salivary gland function at acute time points. Since the serous acinar cells of the Von Ebner gland contribute to the secretion of stimulating components of saliva including amylase and lipase, loss of these cells would impair the digestion of carbohydrates and fats in the oral cavity and impair the ability to taste at both circumvallate and foliate papillae. Similar results were also reported by many authors who indicated that apoptosis has a more important role in salivary gland dysfunction after radiation exposure [19-21]. In contrast, other studies have indicated that there is a noticeable decrease in the secretion and flow of the saliva because of radiation without a significant increase in apoptotic cells [22,23]. Our result showed different changes in the form of acinar atrophy with loss of gland architecture which being replaced by either fibrous or adipose tissue with extravasation of blood, this gives the impression of a different behavior of reaction in both epithelial tissue and connective tissue. Other evidence in our results is the presence of a mitotic figure with an increase in acinar cell size, which is consistent with the immunohistochemical findings of PCNA positive cells with variable activity from mild to strong in most irradiated mice which suggests that increased cell proliferation is either a healing activity or formation of neoplasm like-lesion, these results agreed with many investigators [24,25]. Many other researchers agree with the reparative possibilities and say that the increase in intracellular calmodulin gives an indication of cell proliferation and regeneration through DNA repair [26-28]. On the other hand, many researchers have suggested that long-term or late side effects of radiation exposure to head and neck radiation at any age and dose are known to increase the risk for developing malignancy to salivary tissue.

		Levene's Test for Equality of Variances		T-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
PCNA	Equal variances assumed	21.659	.000	-3.528	18	.002**	-39.00000	11.05501	-62.22572	-15.77428
	Equal variances not assumed			-3.528	10.932	.005**	-39.00000	11.05501	-63.35034	-14.64966

Table 3: Independent Samples Test of PCNA expression of both groups.

		Levene's Test for Equality of Variances		T-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
CK17	Equal variances assumed	2.284	.148	-3.578	18	.002**	-.97500	.27246	-1.54742	-.40258
	Equal variances not assumed			-3.578	12.695	.003**	-.97500	.27246	-1.56506	-.38494

Table 4: Independent Samples Test of CK17 expression of both groups.

Cytokeratin is a member of the intermediate filament family found in all types of epithelial cells, where its expression is closely linked to the state of differentiation of epithelial cells and their secretory function. In this study, the cytokeratin staining pattern in the serous gland of the control group was either diffuse or concentrated at the base of the cells. These cytokeratin distribution patterns are thought to be related to the functional activity of the gland, where the diffuse staining pattern indicates the resting phase of the gland while the absence of cytokeratin in the luminal part correlates with the secretory state with active ion exchange at this part, and this result is consistent with some researchers [29,30]. Von Ebner acinar cells from the irradiated group in this study revealed distinct diffuse immunoreactivity for CK17 in both acinar and ductal cells, which is likely because the cell is in a state of dysfunction and has stopped secreting saliva with widespread cytokeratin accumulation. Many irradiated sections also showed a strong expression of cytokeratin, concentrated in the luminal part of the cell cytoplasm, and decreased in the basal part, where it interferes with the secretory activity of parenchymal cells leading to hypo-salivation, these results agreed with the result concluded by many authors [31,32]. In conclusion, strong expression of CK17 is indicative for the impaired secretory function of that gland and reflect the radiation sensitivity of serous acini.

Acknowledgment

I extend my sincere thanks and greetings to my beloved son, Dr. Omar Sherif, Faculty of Medicine, Cairo University, for his wonderful efforts and planning, which supported me to complete this research.

Author Contributions

Mashael Al-Qahtani, Sherif Hassan, and Ibraheem Bamaga reviewed the literature, wrote the manuscript, and revised it critically. The authors have read and approved the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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