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Radiation effects on submandibular gland of the rat. Stereological and ultrastructural study

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The chronic effect of 2000 rd (20 Gy) of X-rays locally applied to the submandibular gland of Wistar young-adult male rats, was studied by stereology, and light and electron microscopic observations. They demonstrated that degenerative changes were observed during the first months after irradiation. Atrophic processes developed from 6 months onwards. In the acinar compartment, atrophy was progressive from the first months post-irradiation up to 9 months post-irradiation. Ductal cell membranes and mitochondrial damage were clearly detected at ultrastructural level. Nuclear fragmentation and secretory granules alterations were also frequently seen in the acinar compartment.

Strahlenwirkung auf die Unterkieferdrüse der Ratte. Stereologisehe und ultrastrukturelle Studie

Die Unterkieferdrüsen junger, erwachsener, männlicher Wistar-Ratten wurden einer lokalen Röntgenbestrahlung mit 2000 rd (20 Gy) unterzogen, und der chronische Effekt wurde stereologisch sowie licht- und elektronenmikroskopisch untersucht. Während der ersten Monate nach der Bestrahlung zeigten sich degenerative Veränderungen. Ab dem sechsten Monat entwickelten sich atrophische Prozesse. Im azinösen Teil zeigte sich vom ersten bis zum neunten Monat nach der Bestrahlung eine progressive Atrophie. Im ultrastrukturellen Bereich wurden eindeutige Schäden an den Kanalzellmembranen und den Mitochondrien nachgewiesen. Im azinösen Teil fanden sich auch häufig Kernfragmente und Veränderungen an den Sekretgranula.

Influence des rayons sur la glande sous-maxillaire du rat. Etude stéréologique et microscopique

La glande sous-maxillaire de jeunes rats wistar mâles adultes était irradiée avec 2000 rd (20 Gy) de rayons X. Les effets chroniques étaient étudiés par la stéreologie et à l'aide du microscope classique et électronique. Las observations ont montré des altérations dégénératives se produisant pendant les premiers mois après l'irradiation. A partir du sixiéme mois, des processus atrophiques se développaient. Dans la région acineuse, l'atrophie était progressive dès les premiers mois jusqu'au neufiéme mois après l'irradiation. Au niveau microscopique, on a observé des lésions aux membranes cellulaires des canaux et aux mitochondries. Dans la région acineuse, les framentations nucléaires et les altérations des granules sécrétrices étaient fréquentes.

Introduction

Several authors [2, 7, 16, 20], described the alterations produced by irradiation of rat submandibular glands up to 2 or 3 months post-irradiation. Electron microscope studies were performed to describe irradiation damage in the parotid gland [16–18].

As salivary glands are often irradiated during treatment of pharyngeal and oral cancers and such irradiation frequently produces xerostomia and sialadenitis, the studies of the effect of irradiation on salivary glands of mice and rats may contribute to evaluate the changes produced in salivary glands and to correlate them with pathological features observed.

A study comprising light and electron microscopy and quantitative evaluation of the salivary glands of animals that have reached sexual maturity has been carried out up to 9 months post-irradiation.

Materials and methods

Sixty young-adult Wistar male rats, weighing 250 g, were used for this experiment. The area located 2 to 3 mm from the clavicle up to the line that passes between the maxillary angles, was irradiated with a single dose of 2000 rd (20 Gy) of X-rays.

The animals were placed ventral side up in a lead cage, that shielded the body. The head of the rat was shielded by another lead cage smaller than the first one and a small window exposed the area to be irradiated.

Single doses were delivered by a deep-therapy X-ray Philip machine at 220 Kv, 14 mA with added filtration of 2 mm Al, STD 22,8 cm.

The rats were anesthesized with sodic pentobarbital (Embutal, Parafarm Lab.) given intraperitoneally before irradiation (15 mg/kg body weight). No differences in feeding and drinking habits were observed in irradiated and non-irradiated animals.

The submandibular and sublingual glands were separately dissected free from surrounding tissues, and weighed immediately. The glands were fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylineosin and Gomori's trichrome stain, using Mayer's hematoxylin for nuclear staining. Three slices from each gland were mounted. Irradiated and non-irradiated glands were simultaneously processed.

A semi-automatic analyzing system (Kontron) was used to quantitatively analyze glandular components. Intercalated and striated ducts were excluded from this evaluation due to the irregular distribution observed in the tissue under study.

Data obtained at 3,5 and 9 months post-irradiation were analyzed in this work.

Volume density and surface density were the parameters used in this study. The general principles for the correct use of these parameters have been extensively described by Weibel, Kistler and Scherle [20].

For the electron microscopical study, 2 animals were sacrificed monthly from 1 to 7 months after irradiation. Two sham irradiated animals were used as controls for each time.

The glands were fixed immediately after sacrifice by immersion in Karnovsky's fixative solution [11]. Small tissue pieces were dehydrated in alcohol and embedded in Maraglas. Ultrathin sections were stained with uranyl acetate and lead citrate and photographed in a Philips 300 electron microscope.

Results

Stereologic measurements

Tables 1 and 2 summarize the values of the various parameters evaluated in this study.

Non irradiated submandibular glands show a net weight increase of 25%, measured between 5 and 11 months of age. The rate of weight increase was almost uniform during this period.

Irradiated glands: At three months post-irradiation, X-rays produced a 5% weight decrease in salivary glands. Less volume was occupied by acini and granular ducts when compared to non-irradiated glands. An important reduction of about 35% was measured for acini and a reduction of 16% was calculated for ducts. Acinar surface density decreased 20%. No changes were measured in ductal surface density (Figure 1).

Post-irradiation time	Glandular weight (g) Control	Irradiated
3 months	0.278 ± 0.004	0.264 ± 0.005*
5 months	0.312 ± 0.002	0.255 ± 0.006*
9 months	0.361 ± 0.15	0.155 ± 0.005*

* Differences between control and irradiated samples are statistically significant (Student's test, probability 0.05).

Table 1. Submandibular gland weight values 3, 5, and 9 months post-irradiation.

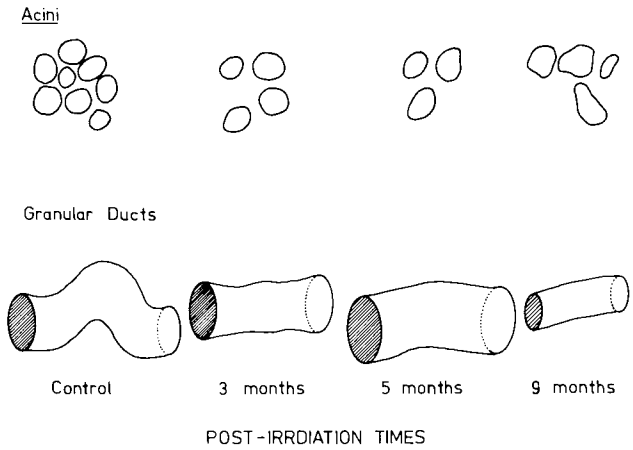

Figure 1. Volume density variations of submandibular gland constituents 3, 5 and 9 months after irradiation.

At five months post-irradiation, glandular weight was reduced by 18%. The reduction of volume density, number and surface density was recorded for acini. An increase in volume density of duct compartment and a decrease in the number and surface density of ducts was measured (Figure 1).

Nine months after irradiation, the gland weight was half that of non-irradiated ones, and a reduction in surface density of duct compartment was also detected. The number of acini was half that of the control values and a marked reduction was also observed in ducts. Acinar surface density increased by 15% (Figure 1).

Histological findings

The submandibular gland exhibited a mild cytoplasmic vacuolization at 3 months post-irradiation, with a moderate

Parameter	Post-irradiation time	Acini Control	Irradiated	Granular ducts Control	Irradiated
Volume (%)	3 months	81.22 ± 3.21	66.29 ± 2.71*	15.39 ± 1.16	13.05 ± 1.53
	5 months	82.31 ± 1.54	56.91 ± 9.67*	15.08 ± 3.24	20.73 ± 1.82*
	9 months	76.77 ± 1.21	60.27 ± 0.81*	18.13 ± 3.90	14.27 ± 2.25
Calculated weight of gland components	3 months	0.255	0.175	0.042	0.034
	5 months	0.256	0.145	0.047	0.052
	9 months	0.277	0.093	0.065	0.022
N°/mm²	3 months	1286 ± 76	829 ± 107*	135 ± 5	108 ± 34
	5 months	1414 ± 136	675 ± 85*	131 ± 10	118 ± 59
	9 months	1124 ± 63	507 ± 57*	128 ± 34	81 ± 5*
S/V (cm^{-1})	3 months	1.10 ± 0.04	0.85 ± 0.01*	0.29 ± 0.04	0.29 ± 0.05
	5 months	1.03 ± 0.12	0.91 ± 0.02	0.35 ± 0.02	0.31 ± 0.08
	9 months	1 ± 0.03	1.14 ± 0.12	-.36 ± 0.08	0.32 ± 0.03

* Differences between control and irradiated samples are statistically significant (Student's test probability 0.05).

Table 2. Quantitative data from irradiated and non-irradiated submandibular glands.

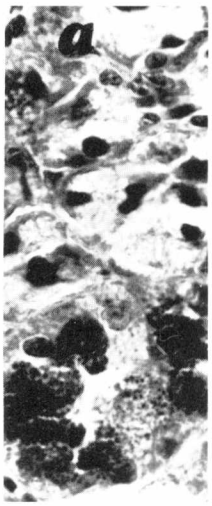

Figure 2

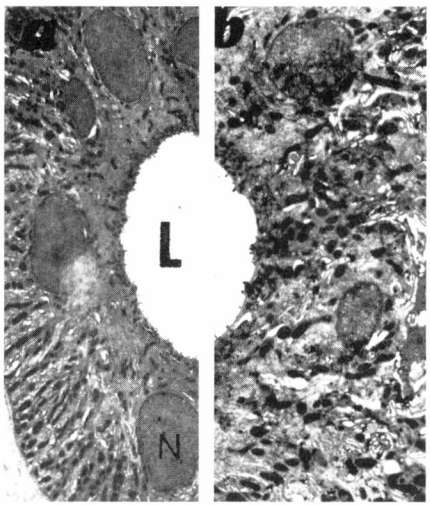

Figure 3

Figures 2a and 2b. Light microscopy: Microphotographs of submandibular glands: a) Non-irradiated gland. b) 3 months after irradiation with 2000 rd (20 Gy). Vacuolar degeneration of the granular duct cells and dilated interacinar spaces between acini are seen. Gomori's trichrome stain and Mayer's hematoxilyn × 160. – Figures 3a and 3b. Electron microscopy: a) Striated duct showing deep infoldings of the cell membrane and many mitochondria in a parallel array. Conspicuous nuclei occupying the central portion of the cell (N), lumen (L). × 1600. b) Striated duct, 4 months post-irradiation. Observe the different width, length and disorganization of the infoldings, alterations in mitochondria and nuclei. × 2000.

degree of pleomorphism among the acinar cells. Some isolated chronic inflammatory foci were observed. During the first 5 months post-irradiation, the vacuolar degenerative changes predominated. At 5 months post-irradiation, the acinar mass was greatly reduced. Some cells showed a disrupted cellular membrane and the secretory material formed some dense masses. It was possible to observe some binucleated cells and nuclear pleomorphism. Some nuclei looked pyknotic and others enlarged. Some acini lose contact with each other and were surrounded by fibrous tissue (Figures 2a, b).

The granular ducts appeared degranulated at 3 months post-irradiation. Granular and striated cells presented vacuolated cytoplasm, which in some sites made the lumen collapse. In others, an amorphous material filled the lumen of the striated ducts. These changes were not uniform throughout the gland, specially at 9 months post-irradiation and two different aspects could be described: Close to the hilium, atrophic images of ducts and acini were observed with large spaces occupied by fibrosis. Nevertheless, at the opposite side of the gland, some ducts had recovered the secretory granules and the acini looked normal, but some anysocariosis and anysocytosis were observed.

Ultrastructural injuries

Important changes were induced by X-irradiation on the ultrastructure of the salivary glands:

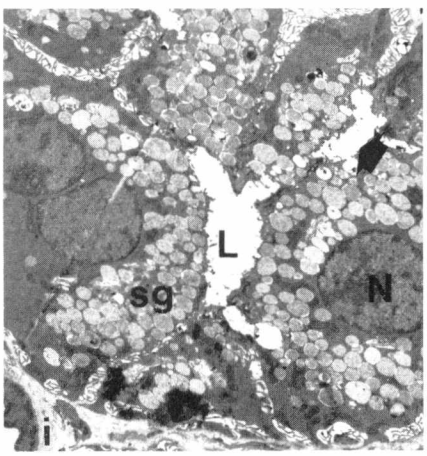

Figure 4

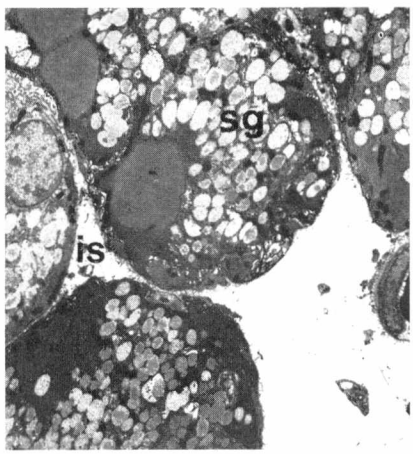

Figure 5

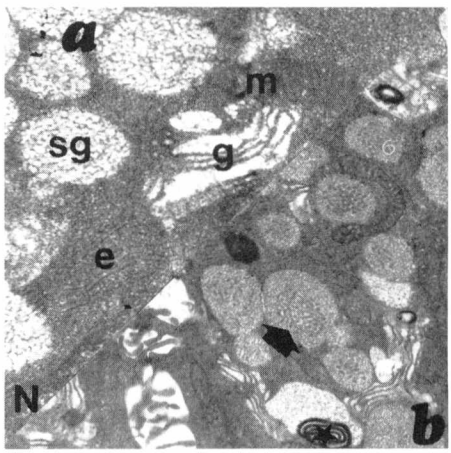

Figure 6

Figure 4. Normal acini. Note the lumen (L), secretory canaliculi (➡), and acinar cell nuclei (N), secretory granules (sg), endoplasmic reticulum area (e), interacinar space (I). × 1500. – Figure 5. Irradiated acini 3 months after irradiation, showing dilated interacinar spaces (is) and heterogeneous secretory granules (sg). × 1400. – Figures 6a and 6b. a) Portion of a normal acinar cell showing dense endoplasmic reticulum (e), some dilated cisternae (g), preceeding the granule formation (sg), mitochondria (m), nucleous (N). × 9000. b) Portion of an acinar cell, one month after 2000 rd (20 Gy) X-rays. Granular material occupying the central portion of dense secretory granules (➡). Numerous hypertrophied Golgi cisternae scattered in the cytoplasm. Some myeline bodies are seen (★). × 7500.

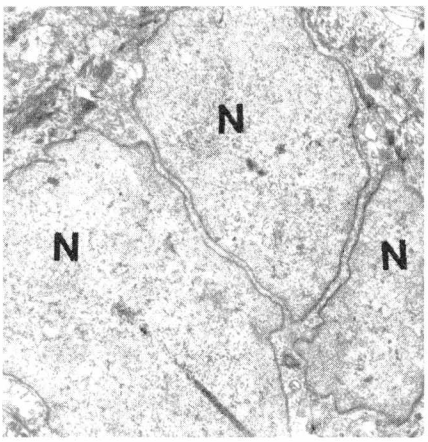

Figure 7

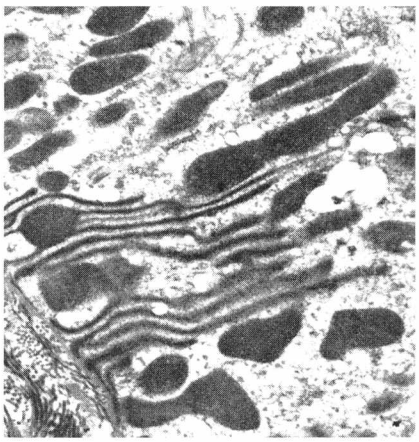

Figure 8

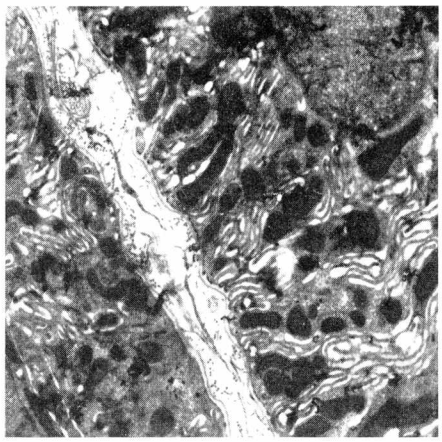

Figure 9

Figure 7. Irradiated acinar cell showing fractionated nucleous (N) and lipid bodies, 3 months after irradiation, ×4200. - Figure 8. Normal striated duct ×8000. - Figure 9. Irradiated striated duct. Observe the alteration in the membrane infoldings and the asimmetric disposition of the mitochondria, two months after irradiation × 5000.

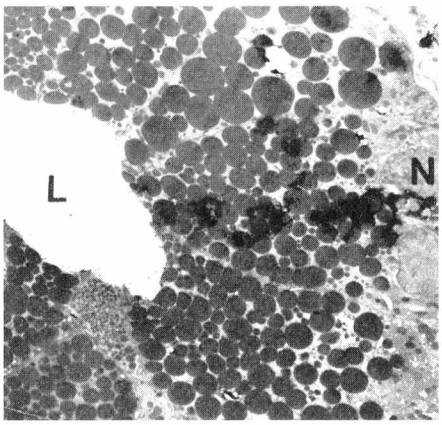

Figure 10

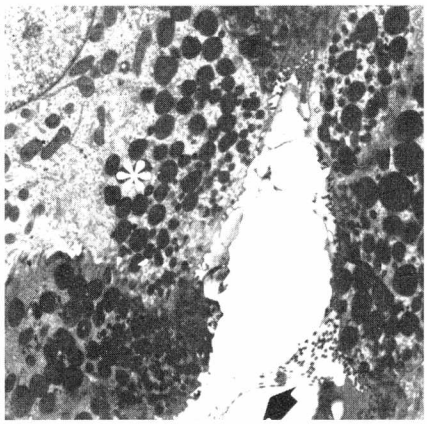

Figure 11

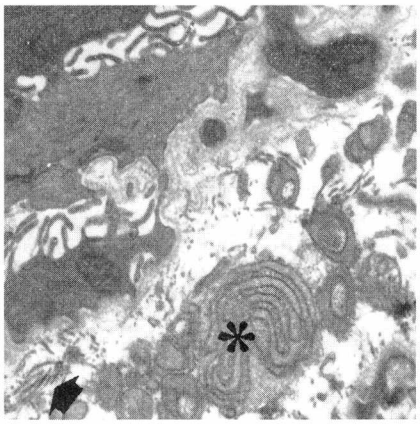

Figure 12

Figure 10. Granular duct showing wide dark granular cells with plenty of large or small dense secretory granules. Lumen (L), nucleous (N). ×1200. - Figure 11. Irradiated granular duct. Note the decrease of the secretory granules (✻) and the alteration in the apical portion of the secretory cell. Fringes of cellular material have merged into the lumen (➡). Two months after irradiation. ×4000. - Figure 12. Interacinar space of an irradiated acini. Collagen fragments (➡), irregular arrays of endoplasmic reticulum (★), widened basal membrane and fragments of cytoplasmic material are seen occupying the interacinar space of an irradiated acini. 4 months after irradiation × 7000.

Collagen fragments and endoplasmic reticulum remnants were observed scattered in dilated interacinar spaces (Figure 13).

Regarding cellular components of *acini,* secretory granules were notably affected exhibiting internally, traces of endoplasmic reticulum-like structures. Dense mitochondria, dilated Golgi cisternae, lipid bodies and fragmented nuclei were frequently observed in acinar cells (Figures 5–8).

Irregular arrangements of *granular duct* cellular components, small number of secretory granules and important alterations of the cell membrane in the apical portion of the cell, were found one month post-irradiation. Disorganized basal lamina and higher density of mitochondria were observed. The lumen of the ducts was often obliterated by fringes of membranes.

Cellular membranes and mitochondria were the most affected structures of *striated duct cells.* Mitochondria exhibited altered shape, higher electron density, and parallel infolding disposition was lost. Basal lamina was widened and disorganized. No fragmented nuclei were observed in ductal cells (Figures 9–12, 14).

Discussion

This study reveals that salivary glands are severely affected by X-radiation. Some interesting quantitative data which correlate well with histological findings are pointed out.

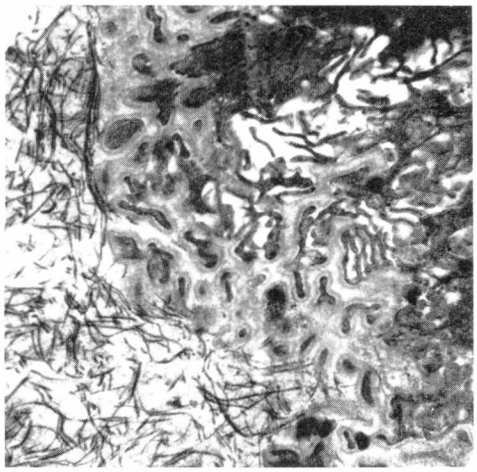

Figure 13. Basal membrane of a secretory duct. Observe the width and disorganization of the collagen fragments 3 months post-irradiation.

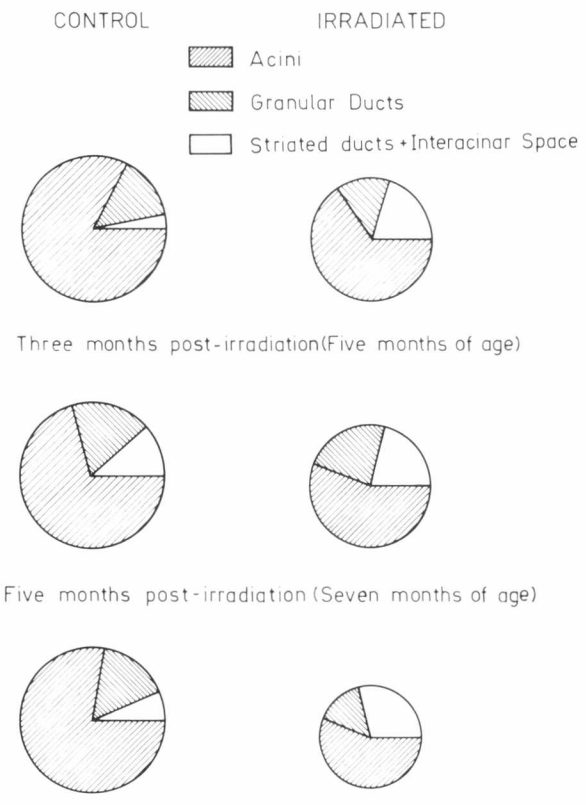

Figure 14. Schematic representation of the changes produced in acini and granular ducts at different post-irradiation times, inferred after stereological analysis.

The acinar compartment presented a continuous decrease in volume density and number, that could be interpreted as an expression of atrophic processes occurring after irradiation, although at 9 months, a slight recovery could be suggested.

Previous descriptions of granular ducts regarded them as being particulary convoluted and longer in males [13], with irregular cross sections images. Considering this and our own results, granular ducts would react increasing their diameter and either shortening their course or becoming straighter. These characteristics were evident 5 months after irradiation but 9 months after the onset of the experiment, ducts would become shorter and the diameter markedly decreased, because atrophic processes were developing, but later than in the acinar compartment.

Degenerative changes in irradiated salivary glands have been observed by other authors, both with light and electron microscopy [2, 15, 17]. A decrease in the weight of acinar components about 30%, 5 months post-irradiation, and about 21%, 9 months post-irradiation was found in this experiment, coincidently with Van den Brenk, Sparrow and Moore findings [19], *despite the different conditions used.*

The stereological study showed that acinar volume density decreased in irradiated specimens. However, this variation is less marked at 9 months post-irradiation, indicating the possibility of a regenerative process.

In our observations the presence of normal acini near the granular ducts filled with granules, may indicate that regeneration from ductal cells can be suggested. Glucksman and Cherry [8] postulated that regeneration from X-irradiation injuries is not likely to occur via the multiplication of duct or intercalated duct cells, but by the replacement of acini by means of their own reproductive activity.

At ultrastructural level it has long been known that acini were the first constituents that showed morphological changes caused by irradiation. Nevertheless, injury to the cell membrane of intralobular ducts was seen only after 2 months post-irradiation. Irradiation treatments have been reported to produce increased viscosity of saliva and acidic pH [3]. This could be attributed to the damage induced by irradiation to ductal cell membrane. Several authors have associated the cell membrane with water metabolism and ion transport in other epithelia and in salivary gland striated ducts [10, 14]. Membrane injury and an uncontrolled accumulation of Ca^{2++} can be the common pathway to cell death [9].

Fragmented nuclei, lipid inclusions and deeply altered mitochondria were observed in acinar cells one month after irradiation. Klein Szanto et al. [12] and other authors [4, 5], described these features in other irradiated tissues. Neither fragmentation nor lipid inclusions have been noticed in ductal cells.

Castle, Jamieson and Palade [1], described the normal pattern of acinar granule formation of rabbit parotid gland. This formation begins in the endoplasmic reticulum and Golgi apparatus. Abnormal acinar secretory granules could be ascribed to the damage of endoplasmic reticulum and Golgi apparatus observed in the specimens.

The presence of wide clear spaces with collagen disorganization could be observed with light and electron microscopy. Stereologic calculations support these observations, although no replacement of collagen fibers was likely to occur until 7 months post-irradiation. Later on, some portions of the gland were filled with collagen structures.

These results demonstrated that both acinar and ductal structures of submandibular gland of the rat are injured by X-rays. An earlier acinar damage was detected and was followed by ductal cell injury. This difference may be due to the length of time required for the cellular injury to become manifested.

An attempt is made to graphically interpret the numerical results (Figure 14).

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