Acta Odontologica Scandinavica. 2014; 72: 386–391

informa
healthcare

ORIGINAL ARTICLE

X-ray irradiation alters the actin cytoskeleton in murine lacrimal glands

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Abstract

Objective. The aim of this study was to evaluate the effects of X radiation on the distribution of filamentous actin (F-actin) in the mouse exorbital lacrimal gland. ***Materials and methods.*** Mice were divided into groups that received no radiation ($n = 6$) or one single exposure of 36 mGy of X radiation ($n = 12$). The animals were sacrificed after 4, 8 or 24 h. The lacrimal glands were stained with Hematoxylin/Eosin or Rhodamine-phalloidin and the filamentous actin arrangement was analyzed by confocal microscopy. ***Results.*** After 4 h of X-ray exposure there was an apparent increase in acini area and a decrease in the cortical F-actin content in secretory cells. This effect decreased gradually over time, returning to values close to the control after 24 h. ***Conclusion.*** This study shows that a 36mGy diagnostic X-ray dose affected reversibly the mouse exorbital lacrimal gland, suggesting that radiation used in diagnosis may induce changes on cell morphology due to actin remodeling.

Key Words: *X-ray, conventional radiography, filamentous actin, exocrine gland, biological effects*

Introduction

Epidemiological studies show that the population is constantly exposed to low doses of different kinds of radiation and that ~15% of this exposure comes from artificial sources [1]. It was shown that almost all of the artificial radiation exposure is due to medical purposes, largely from diagnostic procedures such as dental X-rays or Computer Tomography; the absorbed dose (Gy) may vary widely, according to the procedure and equipment employed, reaching values as high as 500 mGy [2–4]. Until now, there are no safe radiation levels established and some reports even suggest a correlation between X radiation exposure for diagnostic purposes and the development of late-onset tumors [5].

Through the transference of energy to biological molecules, radiation can cause reversible or irreversible damage in cells and tissues by modifying gene expression [6], inducing genomic instability [7], changing cell morphology [8], and affecting the cell cytoskeleton [9]. *In vitro* studies showed that a low dose of IR is able to change signaling pathways [10], the cellular microenvironment [11], as well as the function of non-irradiated cells, which are considered bystander cells [12].

Patients under radiotherapy for head and neck cancer frequently present severe dry-eye syndrome, suggesting that a high dose radiation might lead to hypofunction of lacrimal glands [13]. These changes usually result in a reduction of the ocular surface protection and a higher susceptibility to opportunistic infections [14]. Stephens et al. showed that a low dose radiation is able to induce an acute reduction of tears in rhesus monkeys due to an induction of apoptosis of lacrimal gland serous cells [15]. Besides the induction of apoptosis, high doses of radiation affects cytoskeletal proteins in exocrine glands, which in turn might impair the exocytic process [16].

Actin is one of the most important cytoskeletal proteins, playing a role in the maintenance of the cells framework and stability [17]. It is present in cells as a cytoplasmic pool of globular (G) actin, which polymerizes to form the filamentous (F) actin.

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(Received 8 February 2013; accepted 17 September 2013)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2013.847488

Many actin-binding proteins regulate the assembly and disassembly of actin filaments under specific stimuli, in a very dynamic process [18], and some of the regulatory proteins are redox-sensitive [19]. Actin filaments form the main structural element in the cell cortex and are also implicated in the compensatory membrane retrieval that follows the exocytic process [20].

Therefore, it is important to understand the morphological changes promoted by a dose X-ray exposure originated from diagnostic procedures in potentially affected organs. Due to the importance of the actin cytoskeleton for the secretory process of exocrine glands and the fact that X-ray radiation is able to affect the actin framework, the aim of this study was to evaluate the short-term effects of a diagnostic dose of X-ray radiation of 36 mGy in the murine exorbital lacrimal glands, particularly in the acinar cell area and on the arrangement of filamentous actin.

Materials and methods

Animals and experimental procedure

The research protocol was approved by the Ethics in Research Committee of the School of Dentistry of the Federal University of Rio Grande do Sul. Eighteen male Swiss albino mice (*mus musculus*) with 30 days of age were divided into control ($n = 6$) and test ($n = 12$) groups, which received no radiation or one exposure of 36 mGy of X radiation, respectively. A dentistry X-ray unit (70 kVp, 50/60 Hz, 8 mA) was employed for the test group, with 20 cm of focus-animal distance. For immobilization purposes, the animals were placed in a plastic tube and the entrance surface dose was determined with an ionization chamber (Victoreen 450P, Moedling, Austria).

The dental appliance used had a 1.5 mm aluminum filter and the ionization chamber is calibrated in the laboratory reference biannually. The check was made with a standard dosimetry (tablets of CaSO4) (SAPRA-Landauer, São Carlos, Brazil). Twenty consecutive exposures of 0.6 seconds were performed with 20 cm focal distance from the animal resulting in the total dose of 36 mGy (1.8 mGy by exposure).

Mice were anesthetized with ketamine (30 mg/100 g of body weight, i.m.) (Ketalar, Parke-Davis, São Paulo, Brazil) and xylazine (2 mg/100 g of body weight, i.m.) (Rompum, Bayer S.A., São Paulo, Brazil) and exposed to X-ray and sacrificed by cervical dislocation after 4, 8 and 24 h. All mice of the control and experimental group were handled in the same way, except for the x-ray exposure.

Exorbital lacrimal glands were removed fixed in 2% formaldehyde solution in 0.1 M phosphate buffer (PB, pH 7.4) for 6 h at 4°C and cryoprotected in 30% sucrose solution in PB at 4°C during at least 24 h. Glands were embedded in O.C.T. compound (Sakura, Torrance, CA) and cut using a cryostat (Jung, Leica, Deerfield, IL). Seven micrometer-thick slices were positioned in gelatin-coated slides, heated for 1 h at 37°C and kept at –20°C until Hematoxylin-Eosin (H&E) and filamentous (F) actin staining.

Absorbed X-ray dose calculation

Radiation exposure equals the total charge of ions (positive or negative) produced per unit of dry air by a given amount of ionizing radiation. In SI units, exposure is usually measured in terms of coulombs (C) per kilogram, but it is also commonly measured in units of roentgens (R), where $1\ R = 2.58 \times 10^{-4}$ C/kg. The unit of dry air is denominated air kerma, where kerma is an acronym for 'kinetic energy released in material' [21] and it represents the sum of the kinetic energy of all of the charged particles liberated per unit mass of a material by an amount of ionizing radiation. When that material is air, the kerma is referred to as air kerma. Thus, whereas exposure measures electric charge produced in air per unit mass from an amount of ionizing radiation, air kerma measures its energy produced in air per unit mass. The air kerma measures were performed with a 20 cm distance to the focus of X-rays source and the detector field was totally immersed in the beam. In this way, using the conversion factors of the ICRP 74 [22], it was possible to close up the measured dose (36 mGy) as similar to those applied in the skin (entrance surface dose).

H&E and F-actin staining

For H&E staining the slices were hydrated in PB, stained with Hematoxylin (1 min), washed in distilled water, stained with eosin (30 s), washed with PB and mounted with glycerol-carbonate solution. The slices were observed under in a Leitz Aristoplan light microscope (Leica, Wetzlar, Germany).

For the F-actin staining, Rhodamine-phalloidin (Molecular Probes Inc., Eugene, Oregon, USA) was employed, according to the manufacturer's instructions. The slices were hydrated in PB and stained with Rhodamine-phalloidin (1:100, 1 h, RT). Sytoxgreen (Molecular Probes Inc.) was used for nuclear staining at a 5000× dilution, for 10 min, RT. After washing, the slices were coverslipped, analyzed under a confocal microscope (Nikon PCM2000, Tokyo, Japan) and the pictures were mounted with Adobe Photoshop 6.0 (Adobe, San Jose, CA) and Deneba's imaging software (Deneba Software, Miami, FL). No changes were performed in the photomicrographs.

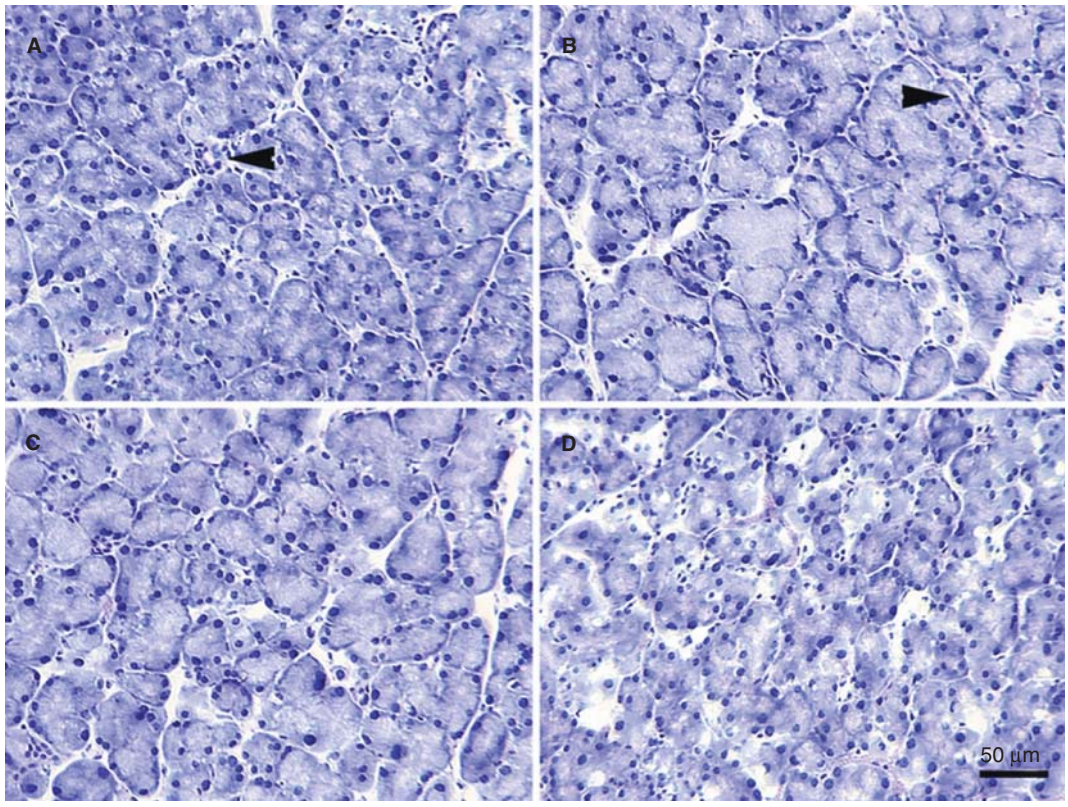

Figure 1. Haematoxyllin and Eosin (HE) staining of the exorbital lacrimal gland from a control mouse (A) and from irradiated mice 4, 8 and 24 h after exposure (B, C and D, respectively). The arrowheads point to intralobular ducts. Magnification bar =50 µm.

Morphometric analysis of acinar cells

Glands stained for F-actin were used for this analysis, because the observation of individual acinar cells was possible in those preparations. The area occupied by acinar cells was measured using the Image Pro-Plus 3.12® (Media Cybernetics, Silver Spring, OH). At least 27 photographs comprising an area of 0.25 mm^2 were analyzed for each group (3 mice, 3 sections of each gland, 3 photographs from each section at a 40× magnification). In each photograph, ~90 individual cells were measured. Analysis of variance followed by Tukey's procedure was used for the statistical analysis. The results were expressed as mean ± SD and considered significantly different at $p < 0.05$.

Results

Approximately 80% of the exorbital mouse lacrimal gland consists of serous acinar cells (Figure 1A). The duct system is quite simple and intralobular ducts are formed by a simple cuboid epithelium (Figure 1A, arrowhead). The glandular stroma is scarce, containing connective tissue cells. No differences were observed between animals from the control group. After 4 h of X-Ray exposition, the lacrimal glands showed an apparent increase in acinar area (Figure 1B). This effect was still observed after 8 h (Figure 1C), disappearing 24 h after the exposure (Figure 1D).

In order to study the distribution of F-actin in acinar cells, Rhodamine-phalloidin was employed. In control animals F-actin was mostly distributed in the cell cortex, just beneath the plasma membrane, and also around intercellular canaliculi (Figure 2A, arrowheads). Radiation visibly reduced F-actin content in the cell cortex, poorly maintaining intercellular canaliculi (Figure 2B). After 4 h of exposure a discontinuity of polymerized actin in the acinar cell cortex was visible (Figure 2B). This effect was still partially observed 8 h after exposure (Figure 2C), disappearing after 24 h (Figure 2D). The reduction in actin polymerization was accompanied by an increase in cell area, estimated by the measurement of the area occupied by individual acinar cells (Figure 3). Morphometric analysis showed that there was a significant increase of 38% in cellular area after 4 h, lowered to 19% after 8 h and returning to normal values after 24 h (Figure 3).

Discussion

In this study we showed that a 36 mGy X-ray dose caused reversible alterations in the actin cytoskeleton and a temporary increase in area of murine exorbital lacrimal gland cells. These changes might play a role in the secretory impairment of the lacrimal gland

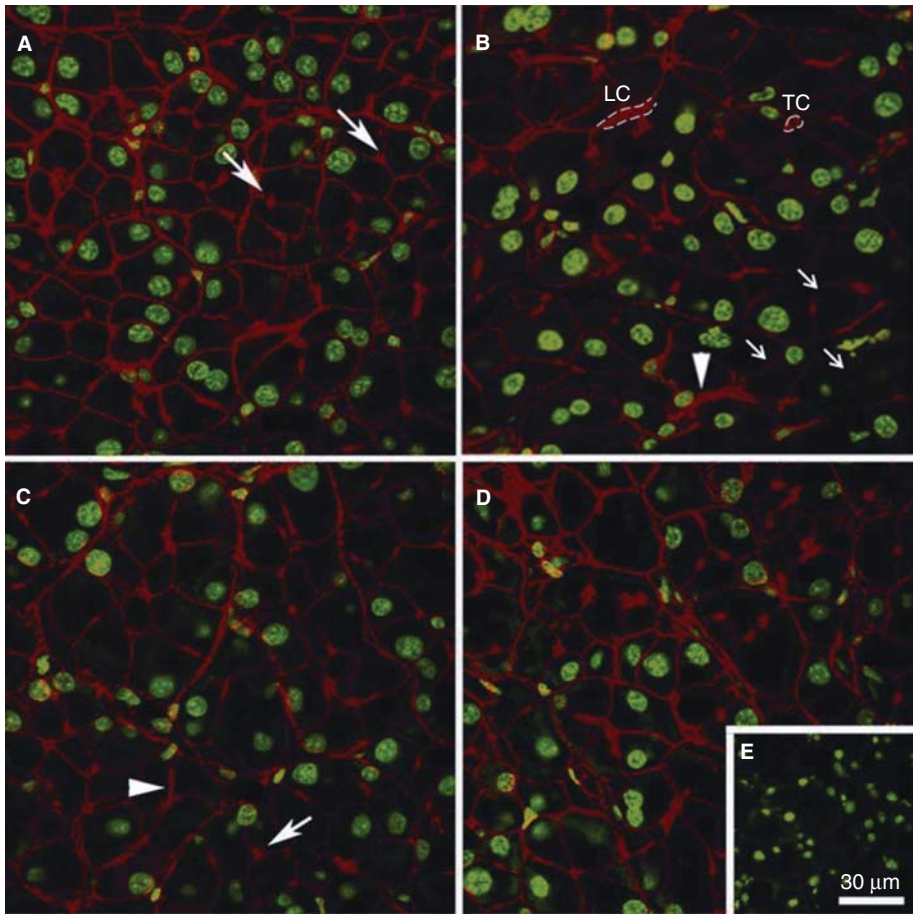

Figure 2. Radiation promotes actin depolymerization in acinar cells of the exorbital lacrimal gland. Rhodamine-phalloidin staining of F-actin in lacrimal glands from a control mouse (A) and from irradiated mice 4, 8 and 24 h after exposure (B, C and D, respectively). (E) Negative control of the reaction, only with nuclear staining using Sytoxgreen. Arrows and arrowheads point to transversal and longitudinal views of intercellular canaliculi, respectively. Open arrows point to gaps in the cortical microfilament network. Line drawing shows transversal canaliculi (TC) and longitudinal canaliculi (LC). Magnification bar =30 μm.

previously observed after a low dose X-ray exposure [15]. Gazda et al. [23] also reported lacrimal gland alterations after exposition to X radiation, but using therapeutic (higher) doses compared to the lower diagnostic dose used in our study.

The total dose of 36 mGy is achieved at some diagnostic procedures in dentistry and it may represent a full-mouth X-ray series [4]. A complete radiographic exam (periapical and interproximal) results in 18 exposures. If there is the need of specific complementation for third molars, the exam can achieve 22 exposures, without the possible repetitions.

The present study suggests that diagnostic radiation doses in the head may affect the lacrimal gland homeostasis *in vivo*, even when the main target of IR is not that gland. Longstreth et al. [5], in a population-based case-control study, recently found an association between increased risk of meningioma and dental X-rays, including full-mouth series achieved many years before. Previous studies from Nolan [24] estimated the dose of radiation on the skin from a full-mouth series to be ~1000–3000 mGy. He also noted the presence of many intersecting radiation lines in the neck and oral cavity, which varied according to the angle at which the X-ray images were acquired. Nolan stated that these points of intersection represented locations where high levels of ionization had occurred. Radiation exposure from dental X-rays decreased over time with the refinement of X-ray machines and films. However, it is still possible to find equipments in use capable of emitting up to 20 mGy doses, corresponding to a single radiograph [2]. This dose can be multiplied when considering a full-mouth series that comprises 10 to ≥22 individual films of all the teeth. Moreover, some clinical investigations require further radiographic assessment, which might affect the same region of the head and neck.

Actin is a cytoskeleton microfilament important to define the cell shape and maintain the cell adhesion. It is also involved in the cell mitoses and in the particles exocytosis. Therefore, the alterations on this filament can bring as a consequence the alteration of the cell behavior.

In this study, actin filaments in lacrimal acinar cells were observed mostly in the cell cortex, just beneath

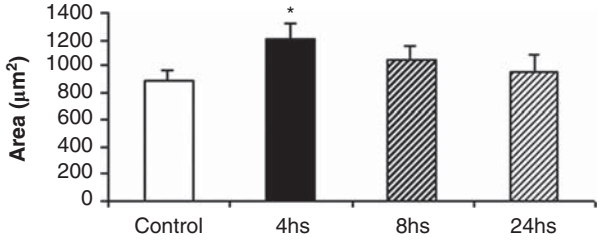

Figure 3. Radiation increases cellular area in acini of the exorbital lacrimal gland. The area (in μm2) occupied by individual acinar cells was measured in photographs taken from glands previously stained for F-actin. Bars represent control and irradiated mice 4, 8 and 24 h after exposure. Data are expressed as mean ± SD. $*p < 0.05$, according to ANOVA and Tukey's post-test.

the plasma membrane. This actin arrangement is essential not only for the maintenance of cell morphology, but also for functions such as endocytosis, exocytosis, and vesicle trafficking [25]. In rabbit lacrimal glands, apical filamentous actin forms a barrier restricting access of secretory vesicles to the apical membrane, and the acini stimulation triggers a transient re-distribution of these apical microfilaments, allowing secretory vesicles to move past this barrier and fuse with the apical membrane [26]. Apparently, a high rate of clathrin-mediated apical endocytosis is also important for this process and depends on the normal actin dynamics. Also in acinar cells, contractile bundles of actin and myosin II assemble around a pool of secretory vesicles, allowing granules fusion and facilitating exocytosis by pushing the contents towards the cellular apical membrane for extrusion into the lumen [25]. More recently, Jerdeva et al. [26], studying the rabbit lacrimal gland, confirmed that the fast remodeling of the actin cortex in response to a secretory stimulus is necessary for exocytosis.

The results showed a partial actin depolymerization in acinar cells, promoted by low doses of X radiation and probably related to the observed increase in cell area. Foskett and Melvin [27] demonstrated that sustained cell shrinkage during sustained elevated $[Ca_2]i$ reflected sustained fluid secretion. Thus, the changes in acinar cells area, the actin network loss and the accumulated granules in the cytoplasm might be involved in an impairment of the normal lacrimal flow. This phenomenon is dependent on diverse mediators that modulate ion channels [28]. It is possible that changes in cortical actin microfilament organization may be markers for intracellular signaling changes that broadly affect cell function.

These effects were observed 4 h after irradiation, gradually disappearing within 24 h. In agreement with this result, Somozy et al. [29] observed in HT29 cultured cells that actin and intermediate filaments were rapidly disrupted following low doses of radiation, with the formation of actin clumps in the cytoplasm. Some *in vitro* studies demonstrated that low doses of radiation affect cytoskeletal proteins, consequently altering cell or tissue integrity [9]. Yin et al. [30] demonstrated that 0.1 mGy irradiation was able to induce the expression of several cytoskeleton-related genes in the mouse brain, but those changes were qualitatively different from those promoted by a 2 Gy irradiation.

There are several reports showing that low dose ionizing radiation can influence the cell response through the activation of signaling pathways and the modulation of gene expression [31]. A possible mechanism is that IR might induce transference of energy to biological molecules, which might cause reversible and irreversible damage in cells and tissues [31]. It is possible that the X-ray dose used in our study might induce the generation of oxidative stress in the lacrimal gland cells with consequent changes in the cytoskeleton regulatory machinery [32]. However, more studies are necessary to test this hypothesis.

Conclusion

In conclusion, we reported that a 36 mGy radiation dose reversibly affected the actin cytoskeleton and area of acinar secretory cells in the mouse exorbital lacrimal gland. Our results suggest that the radiation, even at low dose levels used in diagnosis, may negatively affect the function of this gland.

Acknowledgments

The authors would like to thank Raquel Matos de Oliveira from The Laboratory of Ageing Biology at the Pontifical Catholic University of Rio Grande do Sul (PUC/RS) for the excellent technical assistance. This study was supported by FAPESP. Marcelo Lamers received a scholarship from CNPq.

***Declaration of Interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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