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Effect of low radiation dose on the expression and location of aquaporins in rat submandibular gland

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ABSTRACT

Head and neck cancers are common in several regions of the world and the treatment usually includes radiotherapy. This treatment can generate adverse effects to the salivary flow, with a relationship between the dose and the damage caused. Salivary gland cells are highly permeable to water and therefore, they express aquaporins (AQPs). This study analyzed changes in the expression and location of these proteins and identified morphological changes induced by low radiation in rat submandibular gland. Female rats were divided into control and irradiated groups. Immunohistochemistry analysis allowed confirming the presence of AQP1 in the blood vessel endothelium. Intense and steady labelling granules were also observed in the cytoplasm of submandibular gland ductal cells. In addition, there was AQP5 positive labelling in ductal cells delimiting the lumen of intercalated duct, in the cytoplasm and membrane of acinar cells. Finally, the decrease of AQP labelling in irradiated animal glands validated their radiosensitivity. Thus, the decrease in AQP1 protein levels in the endothelium and AQP5 in gland ductal cells of irradiated animals may have hindered the removal of water from the lumen of ductal cells, inducing a delay in water absorption and triggering a slight lumen increase.

1. Introduction

Salivary glands might undergo changes that lead to hypofunction during the aging process. Besides aging, *diabetes mellitus*; use of antidepressants, antihypertensive and diuretics; as well as radiation therapy for tumorous diseases (Rosa et al., 2008; Jham and Freire, 2006; Lucena et al., 2010; Vieira et al., 2012; Deasy et al., 2010; Liu et al., 2012); or even menopause (Minicucci et al., 2013) and autoimmune diseases such as Sjogren's Syndrome (Jensen and Vissink, 2014), also alter salivary glands function.

Changes in salivary glands and the impact on volume and quality of saliva may have harmful deleterious consequences for the oral cavity in dentate individuals, in which the lack of saliva affects, in particular, the ability of oral tissues to defend itself against external aggressions (Saleh et al., 2015). In partially and fully edentulous individuals, the saliva is being related to defence capabilities and also plays a fundamental role in stabilizing removable prosthetic devices, which is a factor of high importance for health, comfort and ability to chew in these individuals (Nikolopoulou et al., 2013).

Head and neck cancer accounts for 5% of all malignancies and the treatment protocol for this kind of tumours may include surgery and/or radiotherapy of the oral cavity, maxilla, mandible and salivary glands, which can generate numerous adverse effects to the volume and quality of salivary flow (Jham and Freire, 2006; Vieira et al., 2012; Deasy et al., 2010; Liu et al., 2012).

Deasy et al. (2010) report that reduction in salivary function may start around 1 week after initiation of irradiation therapy, gradually returning after 2 years in cases where radiation does not induce severe and irreversible damage to the glands. Wong (2014) reports an index above 90% of prevalence of some kind of injury to the oral cavity after radiotherapy treatment; xerostomia being the most commonly reported sequel. Lastly, Pinna et al. (2015) conclude that ionizing radiation-induced xerostomia may be considered a multifactorial disease dependent on the chosen type of cancer treatment, as well as the radiation dose applied to the tissues.

Aquaporins (AQPs) are water channels that facilitate and regulate water transport across cellular membranes (Verkman and Mitra, 2000). These proteins are expressed in plasma membrane of cellular types involved in fluid transport and they are present in the membrane of intracellular organelles, thus, regulating cell and organelle volumes (Nozaki et al., 2008). AQPs are involved in a great variety of physiological functions, such as secretory function and homeostasis of water

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and solutes, assisting the transepithelial fluid transport. They also participate in many pathological processes such as glaucoma, epilepsy, obesity and cancer (Verkman, 2005, 2009). Acinar epithelial cells of salivary glands show high permeability to the passage of water, and therefore, have AQPs (Delporte, 2013; Aure et al., 2014; Delporte and Steinfeld, 2006; Matsuzaki et al., 2012; Takahashi et al., 2015; Takakura et al., 2007; Susa et al., 2013).

AQP5 has been the most studied protein in salivary glands and some works have shown that this AQP plays fundamental role in the secretion of fluids in this kind of tissue (Li et al., 2006a, 2006b; Delporte, 2013; Aure et al., 2014). Studies with rodents have identified AQP5 in the parotid, sublingual, and especially, submandibular glands. AQP5 is localized in the apical membrane of serous acinar cells in the submandibular gland where it plays a role in primary saliva production (Li et al., 2006a, 2006b). In the cells of submandibular gland ducts, the presence of this AQP is controversial and still requires further investigation (Delporte and Steinfeld, 2006).

The radiation used in the treatment of head and neck cancer causes damage to the acinar cells of salivary glands (Johnson et al., 1993). Moreover, a decrease in AQP5 expression in salivary glands of irradiated mice was observed. This decrease might participate in the mechanisms that lead to salivary flow loss (Delporte, 2013; Delporte and Steinfeld, 2006).

AQP1 is also seen in blood vessels of submandibular glands during natal and post-natal rat development. The immunolocalization of AQP1 in human salivary glands has shown that, besides capillaries, this AQP is also associated to myoepithelial cells. In patients with Sjögren's syndrome, an auto-immune disease with unclear ethiopathology, which presents the hypofunction of lacrimal and salivary glands as symptom, a decreased expression of AQP1 is observed in myoepithelial cells, which suggests that this AQP might be involved with salivary gland malfunctions (Delporte and Steinfeld, 2006).

Despite existing literature, little is known about the role of AQPs in salivary glands, and generally, these studies report contradictory data about the expression and/or location of some AQP subtypes. Moreover, experimental studies are lacking in the background scientific literature that could describe the impact of radiation treatments on the salivary glands water channels and studies diverge in relation to intensity and fractionation of the dose. There seems to be a consensus that there is a relationship between the dose and the damage caused. Thus, to add new information about rat submandibular gland, our study proposed to investigate the pattern of AQP localization and identify possible morphological changes induced by low radiation dose in this gland.

Therefore, we have proposed this research to describe the location of AQPs 1 and 5, and to evaluate the effect of low radiation on these AQPs in rat submandibular gland, correlating it with possible general morphology changes and morphometry of these glands.

2. Material and methods

This study used 12 female *Wistar* rats, 10 weeks old, obtained from the Animal Facility of the *Marilia Medical School - FAMEMA* (Biotério Central da Faculdade de Medicina de Marília). The rats were fed with common solid diet and *ad libitum* water, and had adequate lighting and temperature conditions (12-hour light-dark cycle; 23–25 °C).

2.1. Experimental groups

The animals were randomly divided into two experimental groups (6 animals each), control and irradiated groups. Both the groups, subjected to radiation and the control, were anesthetized with ketamin-xylazine (v/v) 70 mg/kg – 7 mg/kg and both groups were placed in treatment position within a box with abducted limbs and hyperextended neck.

2.2. Radiotherapy protocol

To undergo the radiation procedures, after anaesthesia induction, the animals were fixed to a platform. Anaesthesia was administered to prevent movement during the radiation beam emission period. The neck region was irradiated after placement of a lead cone to shield the path of radiation, then allowing only the submandibular gland irradiation. The rats were irradiated with a 7.5-Gy single dose of ionizing radiation emitted from a linear accelerator, VARIAN 6EX brand, positioned at a 20 cm distance from the skin surface. Tomography of the cervical region of the animal was carried out to ensure that the prescribed dose has been delivered to the target organ, avoiding the exposure of surrounding structures (oral cavity, esophagus and trachea), as much as possible. The rats in the control groups were not irradiated.

Fourteen days after neck irradiation, the rats in the control and irradiated groups were euthanized for the collection of submandibular glands. To minimize pain and suffering during the experiments, all animals were exposed to CO_2 chambers to decrease consciousness and thereafter, they were sacrificed. The whole experiment and the surgical procedures present in this study are in accordance with the recommendations of the Canadian Council on Animal Experimentation and were approved by the Ethics Committee on Animal Use (CEUA) FAMEMA, Protocol 414/15. The research was conducted according to ethical principles in animal research adopted by the National Council for Animal Experiments Control (CONCEA).

During the experimental protocol, a daily monitoring of animals was performed for quantification of food and water intake, as well as animals' body weight.

2.3. Material processing

The submandibular glands were dissected and weighed, and the right-side ones were immediately frozen in liquid nitrogen and stored in an -80 °C freezer, while the left-side ones were fixed in 4% Sorensen in 0.1 M phosphate buffer glutaraldehyde and 2% formaldehyde fixing solution, pH 7.3, for at least 24 h. After fixation, the glands were dehydrated in ascending concentration series of alcohol solutions (70%, 80% and 90%) and kept in 95% alcohol for 4 h. The samples for morphometric analysis were submitted to 1:1, 95% alcohol and plastic resin infiltration for 4 h; infiltration in infiltrating resin for 4 h or overnight, and lastly, inclusion in glycol methacrylate (Leica Historesin – Embedding Kit). Five micrometer thick sections were obtained from Leica RM2245 microtome equipped with a glass blade and were stained using hematoxylin and eosine. The samples obtained for the immunohistochemistry study were fixed by 4% PBS paraformoldehyde immersion for 24 h, dehydrated in ethanol and followed routine for inclusion in Paraplast (Paraplast Plus, St. Louis, MO, USA), and subsequently, sectioned in 4 µm thick sections in a microtome and then collected in silanized slides to be stored until use.

2.4. Morphometric analysis

The obtained slides were analyzed using a microscope (Olympus Microscope BX41 model) coupled to a digital video camera (Olympus, SD 25 model, DP2-BSW-Olympus Software), and the images were submitted to analysis of the following parameters: tissue volume, duct diameter and lumen.

The slides intended for volume morphometric analysis were assessed considering the equivalent percentage of ducts, acini and stroma, obtained by points counting through planimetric counting method, which is based on placing a grid totalling 168 points on the captured image, using the Image-Pro Plus Software.

Two slides for each animal were analyzed, with 6 animals per experimental group, yielding 36 histological fields per animal, in a random manner, while avoiding areas with preparation artefacts. Data were tabulated, achieving a percentage related to ducts, acini and