REVIEW

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HARDERIAN GLAND OF WISTAR RATS REVISED AS A PROTOPORPHYRIN IX PRODUCER*

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ABSTRACT

In 1694 Johann Jacob Harder described, for the first time, the harderian gland, located near the eye in the great majority of vertebrates, and regarded it as a lacrimal gland. This gland has multiple functions that vary according to the animal species. In the present work, we review the literature on the harderian gland of rats, focusing our study on the detection of great amounts of protoporphyrin IX (PpIX) produced by rodent glands. Protoporphyrin IX is a powerful photosensitizer that is widely used for photodynamic therapy (PDT). We also discuss the anatomic and the histological evidence for the presence of PpIX in the harderian gland of Wistar rats. Protoporphyrin IX has been detected in the lumen and in acinar cells of this gland, as confirmed by fluorescence microscopy. These findings together with numerous reports in the literature suggest that the harderian gland could be useful experimental model for studying the photodynamic process.

Key words: Fluorescence, harderian gland, photodynamic properties, protoporphyrin IX, Wistar rat

Harderian gland

While studying *Dama vulgaris* deer in 1694, Johann Jacob Harder discovered a gland that he described as being of the lacrimal type. Since then, much information has been accumulated about these glands, known as Harder's glands or harderian glands. In rats, the harderian gland produces protoporphyrin IX (PpIX), and for this reason has attracted considerable interest as an experimental model for studies on endogenous fluorophores [1]. Sakai and Yohro [45] reported the presence of protoporphyrin in the harderian gland of most groups of vertebrates, including amphibians (anurans), reptiles, birds and mammals, but noted that this compound was absent in bats, cattle, horses, terrestrial carnivores, large primates and humans.

The harderian gland may be of the tubular [cited in 7,40], tubuloacinar or tubuloalveolar type [24], and there are no significant structural variations among the different species of animals.

The harderian gland synthesizes a wide variety of substances that vary according to the different animal species. These substances include: mucolipidic (amphibians), mucoserous (reptiles), serous (snakes) and lipidic (birds) secretions. In mammals, there are three types of secretions, namely: lipids, indolic products and porphyrins, which are characteristic of some rodents, including rats [11,20,22, cited in 40,54].

The diversity of products synthesized by this gland has resulted in several hypotheses [cited in 7] to describe its functions, although no explanation has been fully satisfactory. Indeed, the functions of this gland vary among animal groups and include a role in the immune response, mainly in birds [36, cited in 40], the production of pheromone [35], the formation of a seromucous secretion for lubricating the eye and the nictitating membrane in mammals and amphibians [cited in 7,12], the production of saliva in some chelonians [cited in 40], osmoregulation in reptiles [6], photoprotection in rats by regulation of the incidence of light on the retina [26], photoreception, detected in a series of experiments with newborn rats [59], thermoregulation [cited in 7,15,21,53], and growth factors in rodents [cited in 7,62].

Anatomical aspects

The harderian gland is situated in the retro-orbital region [57] towards the midpoint of the internal space between the eyeball and the extremity of the orbit and the posterior surface bordering the frontal bone [47]. In mammals, the gland is well developed,

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especially in rodents such as rats, hamsters, gerbils, mice and guinea pigs [cited in 7]. Figures 1 and 2 illustrate the topography of this gland in relation to the cranium and orbits of Wistar rats.

The anatomical structure of the harderian gland has been studied in detail for several species of desert rodents [45]. In rats, the harderian gland has an irregular shape, with three fissures [57,58]. One of these fissures contains the optic nerve whereas the other two contain extraocular muscle fibers. Externally, the harderian gland is enveloped by a membrane, which penetrates the gland, dividing it into several lobules. The excretory duct consists of a proximal sinuous part that opens directly into a distal straight ampulla. A single narrow channel drains the contents of the ampulla onto the concave surface of the nictating membrane [14,29].

Histological aspects

Many studies have investigated the histology of the harderian gland. Brownscheidle and Niewenhuis [1] observed morphological differences among the cells that constitute this glandular tissue in rats. In mammals, the secretory tubules of the harderian glands are usually formed by a simple epithelium composed of cuboidal cells. Staining with toluidine blue have allowed the identification of both types of glandular epithelial cells, which have been referred to as A and B cells [46], or type 1 and type 2 cells, respectively [61]. Type 1 cells account for approximately 75% of the columnar epithelium, and are characterized by having and acidofilic cytoplasm and a large number of lipidic vacuoles. Type 2 cells are less common and are characterized by having a basophilic cytoplasm with a small number of large lipidic vacuoles. Binucleated cells are common in both cellular types. Myoepithelial cells may also be present and are generally arranged in parallel with the basal membrane. The tubule lumen frequently contains a reddish-brown secretion with the color being indicative of the presence of porphyrins [5].

Johnston *et al*. [28], while studying the plain mouse *Pseudomys australis,* described a third cellular type, which has as main characteristic a large central nucleus and account for 11 to 29% of the epithelial cells. These cells have also been found in other species of desert rodents [13].

In the numerous reports of harderian gland histology, there has been little mention to the excretory duct. According Djeridane [14], no ducts were observed within the gland. There was a single specialized excretory duct, which is originated in the hilus. This duct is lined by a stratified, cuboidal epithelium and surrounded by thick layers of connective tissue composed primarily of densely-packed collagen fibers.

The interstitium of the harderian gland contains the normal components of connective tissue such as fibroblasts, collagen fibrils, nerves and blood vessels. In addition, mast cells, macrophages, melanocytes, and small aggregates of lymphoid cells, lymphocytes, plasma cells and eosinophils are also present [14].

Ultrastructure of the harderian gland of Wistar rats

In an ultrastructural analysis of the two cellular types present in the harderian gland of Wistar rats, Brownscheidle and Niewenhuis [1] noted that the material found in the interior of the secretory vacuoles consisted of lipids, protoporphyrin crystals and fibrogranular material.

According to Djeridane [14], type A and type B cells of the Wistar rats harderian gland share many structural features. The apical cell border of both cell types is covered with short microvilli. The membranes of neighboring cells are bound together at their apices by junctional complexes. The lateral and basal membranes are smooth but, in some instances, poorly developed cell folds are present on the basolateral surfaces. The nuclei of both cell types are mostly spherical, large, and located basally, with one or two prominent nucleoli. Both cell types contain lipid vacuoles with a maximum diameter of 3.5 μm in type A cells and 2.5 μm in type B cells. However, the vacuoles of type B cells clearly differ from those of type A cells. The former cells frequently contain electron-dense material that at higher magnification appears to be lamellar, and coats the inside of the vacuole membranes, whereas the vacuoles of type A cells commonly contain an electron-dense, ribbon-like material.

In rats, the mitochondria present in type A cells are larger than those in type B cells, and their matrix is relatively hard to see. The rough endoplasmic reticulum is more evident in type A cells. In type B cells, vacuoles filled with secretion and electron-dense vacuoles are present. The mitochondria of type B cells are numerous and are usually spherical or elongated, with a dense matrix that is easily distinguished from the less dense cytoplasm. These

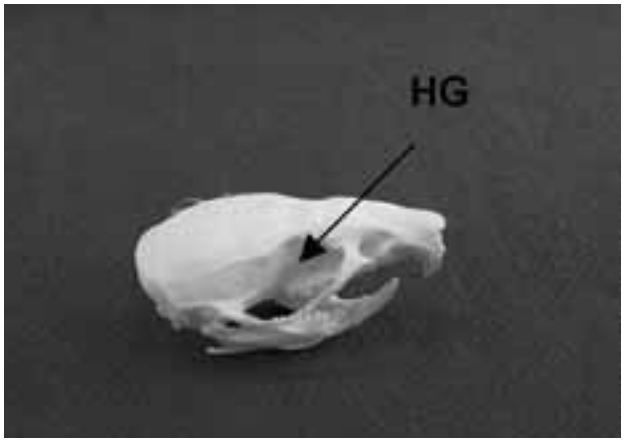

Figure 1. Macroscopic view of a Wistar rat cranium. The arrow indicates the location of the harderian gland.

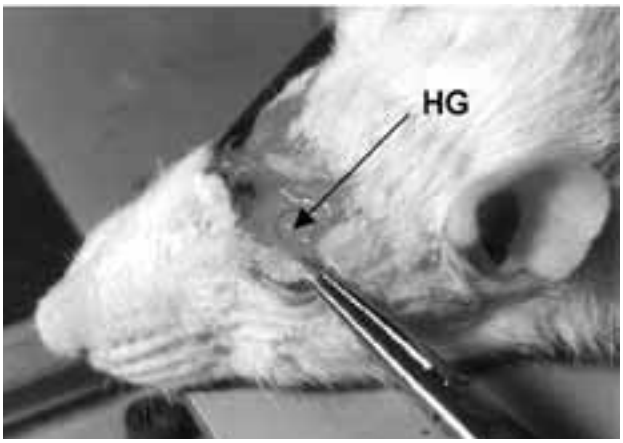

Figure 2. Surgical exposure of the harderian gland of a *Wistar* rat. Note the position of the gland indicated by the arrow (from Reis *et al.* [43], with permission).

mitochondria are smaller than those observed in type A cells and are located around the secretory vacuoles. The vesicles accumulate in the rough endoplasmic reticulum, in a typical "venetian blind" mode [13].

Products synthesized by the harderian gland of Wistar rats

Although many hypotheses have attempted to explain the secretory process of the harderian gland, no satisfactory explanation has yet been provided. Based on an analysis of cells collected from the glandular epithelium of the harderian gland of a golden hamster, Hoffman [24] suggested that apocrine and holocrine secretory mechanisms were involved. In the apocrine mechanism, microscopic fragments of cellular cytoplasm together with protoporphyrin crystals are found in the lumen of acini [6]. In the holocrine mechanism, dead epithelial cells became the gland secretion *per se*. These findings were later confirmed in researches carried out with other species, including Wistar rats [5,14,28,52]. According to Carrieri [5], the detection of porphyrin crystals within the cytoplasm of epithelial cells, accompanied by dilated endoplasmic reticulum vesicles, suggested that the cells were close to death, which would agree with the holocrine mechanism of secretion.

Payne [40] reported cellular debris in the luminar space of the harderian gland. This observation reflects the fact that the harderian gland loses columnar epithelial cells through the physiologic process of cellular renovation. These changes may occur spontaneously, as the animal grows older, or may result from natural or induced hormonal alterations [48,50].

Brownsheidle and Niewenhuis [1], while investigating the ultrastructure of the harderian gland in albino rats, observed that the secretion by the glandular epithelium was continuous, with a constant release of vacuole contents into the gland lumen. Simultaneously, adjacent vacuoles moved towards the apex of the cells and fastened immediately under the plasma membrane, in preparation for the next secretory event. Djeridane [14] also stated that the secretory process was essentially exocytotic, although holocrine secretion may also occur, since nuclear and cytoplasmic debris can also be seen in the acinar lumen.

Derrien and Turchini [11] were the first to report the presence of porphyrin macromolecules derived from immature blood cells in the harderian glands of rats. The gland was initially thought to serve merely as a deposit of porphyrins, which were transported by the blood and deposited in the lumen of harderian gland acini in the form of crystals. However, studies *in vitro* using harderian gland epithelial cells demonstrated that this organ had an enzymatic complex capable of synthesizing porphyrins, particularly protoporphyrin IX (PpIX), from suitable precursors, with subsequent storage of this product in its lumen [9,10,27,30,33,55], along with lipids [58] and indolic products [2].

In mammals, lipids synthesized by harderian gland cells account for around 35% of the dry weight of the gland [58]. Each mature vacuole contains a single lipid droplet surrounded by a simple membranous structure, that in many cases imparts a half-moon format to the amorphous material located between the droplet and the membrane [29,60].

From the biochemical viewpoint, the composition of the lipid secretion varies among species according to the radical groupments present in molecular structure. In mice, pigs and golden hamster, lipids with alkyl (CH_4) and acyl (COOH) groups predominate [cited in 7]. In contrast, in rats and Mongolian gerbils, lipids with an ester group predominate in the vacuoles. In some rat species the vacuoles also contain neutral lipids, such as mono, di and triglycerols, as well as cholesterol phospholipids (phosphatidylcholine, phosphatidylethalonamine and sphingomyelin) and glycolipids, the most frequent of which are cerebrosides [cited in 7].

Other products secreted by the harderian gland include indolic derivatives. These products were initially believed to be synthesized by the pineal gland, but the immunocytochemical demonstration of melatonin in the harderian gland [2,39] has shown that the synthesis of this compound occurs in this gland. López–Gonzáles *et al*. [32] found enzymes in the harderian gland that are involved in the metabolism of indolic molecules, such as N-acetyl transferase (NAT) and hydroxyindole–O-methyltransferase (HIOMT). More recently, a review by Payne [40] reputes to the harderian gland the capacity of synthesizing indoles.

Based on experiments using, radioimmunoassay, high performance liquid chromatography (HPLC) and histoenzymology, Chieffi *et al*. [7] have confirmed that the harderian gland contains all of the components necessary for the synthesis of indole derivatives. Nevertheless, doubts regarding the synthesis of these products by this gland have persisted. Three hypotheses therefore have been proposed to explain the presence of these compound: (1) the indolic molecules found in the harderian gland may be produced elsewhere in the organism and simply accumulate in the gland; (2) the indolic molecules may be produced in the harderian gland, and (3) a combination of both of the first two possibilities.

Djeridane and collaborators [16,17] have reported the presence of indolic products including serotonin N-acetylserotonin, 5-methoxytryptamine, 5-methoxytryptophol, 5-hydroxytryptophol and acetic acid 5-hydroxyindole, in harderian gland cells of rodents.

The harderian gland and the pineal gland differ in their control of the metabolism of indolic molecules. One example is that the synthesis that occurs in the harderian gland appears to be independent of the autonomic nervous system whereas in the pineal gland it is dependent on this nervous system [41]. The synthesis of indolic products by the harderian gland is related to several factors, including sexual differentiation, circadian rhythm, seasonal factors and age.

The concentration of porphyrins in rodent harderian glands varies according to gender and species, and is higher in females than in males. In rats, this difference in concentration is not as great as in other species such as the golden hamsters and plains mouse. Porphyrins and porphyrin-synthesizing enzymes contribute to the biochemical dimorphism of the rodent harderian gland [cited in 7]. The synthesis of porphyrins in the species mentioned above is influenced by sex hormones, as shown by the marked decrease in the formation of these compounds after ovariectomy. The opposite occurs in castrated male golden hamsters, in which there is a marked increase in porphyrin levels [cited in 7,48,49]. Similar results have been reported by other authors [8,24,41,61]. In regard to melatonin levels and sexual differentiation, there is no differences concerning rats, although for hamsters, the female levels are much higher, and castration of prepuberal males leads, not only to increased melatonin production, but also to the replacement of the type A for type B cells, with consequent increase in production of PpIX [18,44].

Chieffi *et al*. [7] observed marked variation in the metabolism of indolic products in rats subjected to artificial seasonal conditions and noted that exposure to long days produced variations in the melatonin concentration. In addition, the levels of melatonin, serotonin, 5-hydroxytryptophol and the activity of hydroxyindole-O-methyl transferase (HIOMT) are all higher during periods when the animals remain in the dark [3,4,13,14,17,25,42,48].

Djeridane [17] evaluated the effect of age on the melatonin content associated with the activities of NAT and HIOMT in the harderian gland. He observed in a comparative study carried out with 22-month-old rats and four-month old rats, that the plasma melatonin concentration decreased with age (from four months old to 22 months old), but there were no changes in levels of this substance in the harderian gland.

Protoporphyrin IX

The presence of several types of porphyrins can be observed in the harderian gland of rats. These

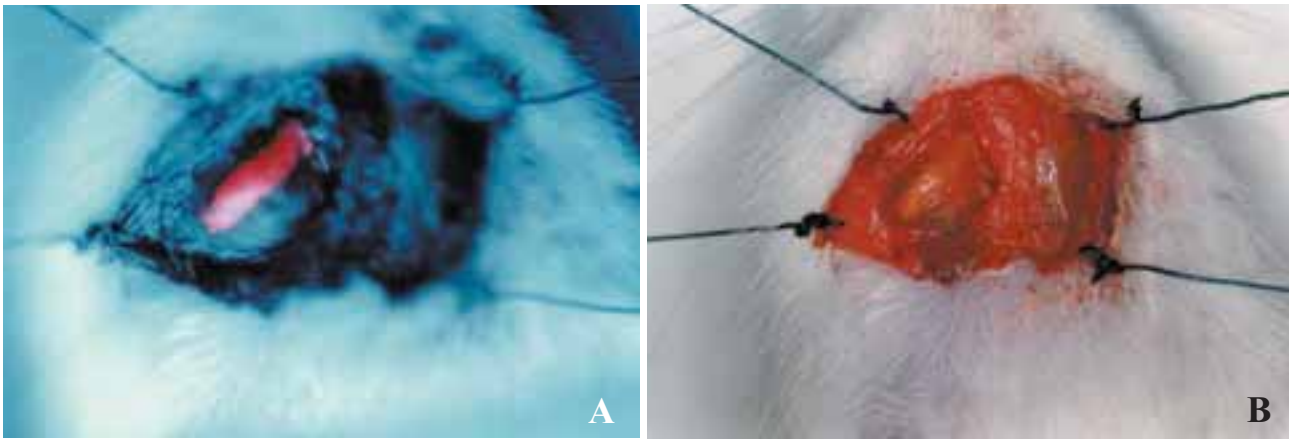

Figure 3. Harderian gland illuminate with ultraviolet (UV) light (365 nm) in **A** showing strong red fluorescence, and under white light in **B**, in which the fluorescence is absent. (from Reis *et al.* [43], with permission).

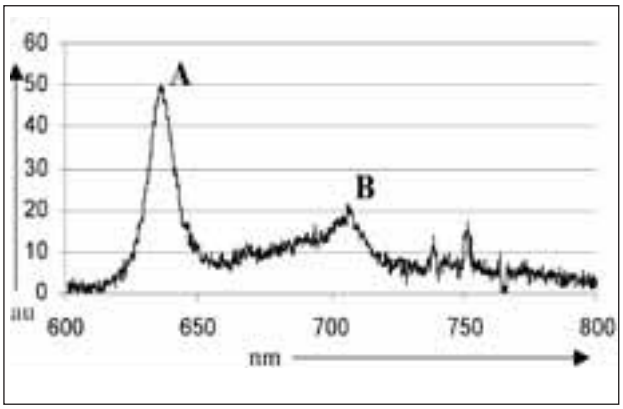

Figure 4. Typical fluorescence spectrum of the harderian gland excited by ultraviolet light. Peak A = 636.2 nm and peak B = 705.8 nm (**au** = arbitrary units).

compounds are distinguished by the different radicals occurring at the carbons located at positions 1 through 8 of the tetrapyrrole ring. The main porphyrins found in the harderian gland of rats are harderoporphyrin, uroporphyrin, coproporphyrin (pentacarboxylic, hexacarboxylic and heptacarboxylic), and protoporphyrin IX [51]. The latter can account for 72% of the porphyrins in the gland [cited in 7]. Many studies have been performed concerning the role of porphyrins in rodent harderian glands, with the objective to understand why these compounds are so abundant in this gland, particularly since this organ has no direct link with the rest of the organism [13]. Protoporphyrin IX is the endogenous product of the rat harderian glands that, on account of its photodynamic action, deserves an in-depth study.

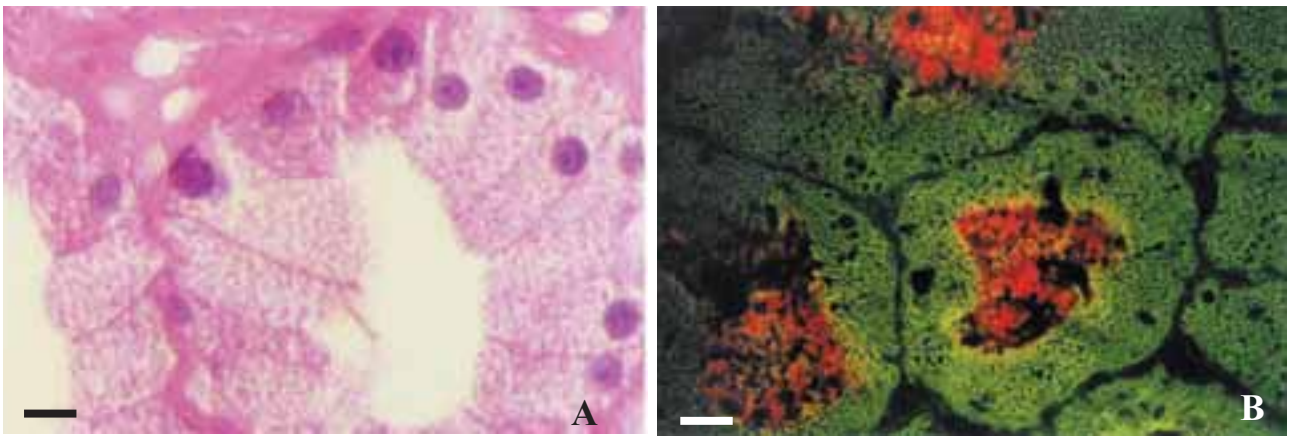

Figure 5. Histological sections of harderian glands. In **A**, a gland fixed in 5 % paraformaldehyde and stained with HE, showing the acinar structure of the gland. Remnants of the secretory contents can be seen in the lumen of the acini. In **B** the gland is under fluorescence microscopy. See the intense red fluorescence caused by protoporphyrin can be seen within the acini. Bars = 15 µm (from Reis *et al.* [43], with permission).

The harderian gland of rats, due to its high concentration of protoporphyrin IX, emits a characteristic fluorescence when irradiated with ultraviolet light [23,43]. This fluorescence was reported by Reis *et al*. [43] after adequate stimulation with 365 nm light (20 watt) from a source positioned 5 cm away from the gland. The intense red fluorescence can be seen with naked eye, as shown in Figure 3A. Figure 3B shows the same gland photographed with white light illumination. The fluorescence was also recorded with an Ocean Optics PC2000 plug-in spectrometer with an optical resolution better than 1.7 nm in the red region. The spectrum confirmed that the red fluorescence was generated by Pp IX based on the presence of the two characteristic peaks (peak A at 636.2 nm and peak B centered at 705.8 nm) as shown in Figure 4.

Light microscopy of the harderian gland parenchyma has shown that the tubules are lined by a single layer of columnar epithelial cells surrounded by myoepithelial cells, with traces of a yellow-brown material corresponding to Pp IX deposits in the lumen (Figure 5A). Fluorescence microscopy has confirmed the presence of Pp IX in the lumen of the acini based on the intense red fluorescence (Figure 5B).

The harderian gland and the photodynamic processes

Photodynamic process is a less invasive optical technique that can be used in medicine as an alternative in the diagnosis and treatment of several types of malignant and non-malignant lesions. This technique is based on the principle that adequate wavelengths of light can interact with non-toxic compounds (photosensitizers, either injected into the organism or of endogenous origin) to generate characteristic diagnostic fluorescence, known as Photodynamic Diagnosis (PDD), or to activate oxygen within the cells which would result in an effective therapy, known as Photodynamic Therapy (PDT) [23]. Contributions towards the understanding of the photodynamic mechanism are important in the areas of biology, chemistry, medicine and physics.

Conventional PDT is based on the activity of an exogenous photosensitizer present in the tissue (tumoral or dysplastic) that is being treated. This photosensitizer, normally a hematoporphyrin derivative (HpD), absorbs photons from adequate wavelengths of light source (generally laser light), and transfers this energy to stable molecules of oxygen (triplet oxygen) that, in turn, become chemically reactive (singlet oxygen). Note that by basic physics principle the triplet – singlet oxygen transition cannot be achieved by direct photon absorption. It instead always demands an intermediate energy conversion provided, in this case, by HpD. The immediate chemical reaction of singlet oxygen with the components of pathological tissue results in the selective destruction of the host tissues [19]. Since the 1980s, PDT has been considered a promising technique for the diagnosis and treatment of certain types of tumors.

Aminolevulinic acid (ALA), a precursor in the biosynthesis of hemoglobin, chlorophyll cytochromes, biliary pigments and other porphyrins, is also widely used in PDT. In this case, exogenous ALA activates protoporphyrin IX synthesis, thereby increasing the concentration of this macromolecule in the organism. ALA enters the blood stream, becomes attached to the surface of the tumoral cells, and plays the same rule as HpD in photodynamic activity [31].

From the viewpoint of physics there is no difference if the photosensitizer is attached to normal or to tumoral cells. The presence of endogenous PpIX in the harderian glands of rats provides a natural and easily accessible experimental model for studies concerning the light involvement (essentially, wavelength and fluence) in the photodynamic process. Although the harderian gland can be useful for studying photodynamic processes since there is no need to inject exogenous substances into the organism, it must be remembered that, in this case, one is dealing with healthy cells, and that many other factors may need to be considered when studying PDT in tumoral tissue.

As shown by Reis *et al*. [43] and in accordance with ethical recommendations for experimental use of animals [56], the exposure of harderian gland to HeNe light (632.8 nm, 8 mW, 45 min, fluence of about 2.7 Joules/mm^2) resulted in cell destruction similar to that seen in cancer tissue treated by conventional PDT. Immediately after exposure to laser light, necrosis of the tubular cells, characterized by fragmentation of the epithelial cells can be observed as shown in Figures 6A and B. After 24 h, there was a marked interstitial edema with granulocytic infiltration in the previously irradiated area and the fragmentation of necrotic epithelial cells was greater (Fig.7A). Remnants of the porphyrin-lipid-complexes were only rarely detected by fluorescence microscopy in the area of necrosis (Fig.7B). The contralateral sham-

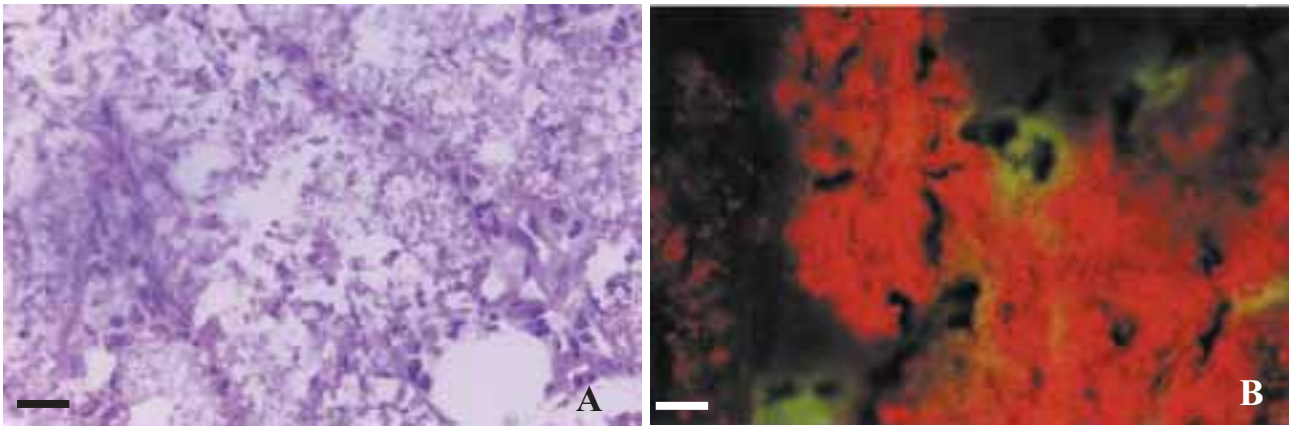

Figure 6. A. Histological section of a harderian gland removed immediately after exposure to laser light: necrosis with fragmentation of the tubular epithelial cells is the predominant feature. HE staining. **B**. Harderian gland under UV light immediately after exposure to laser light. Note the porphyrin-lipid-complexes (red fluorescence) intermingled with necrotic cell fragments, and absence of any inflammatory infiltrate. Bar = 15 µm (from Reis *et al.* [43], with permission).

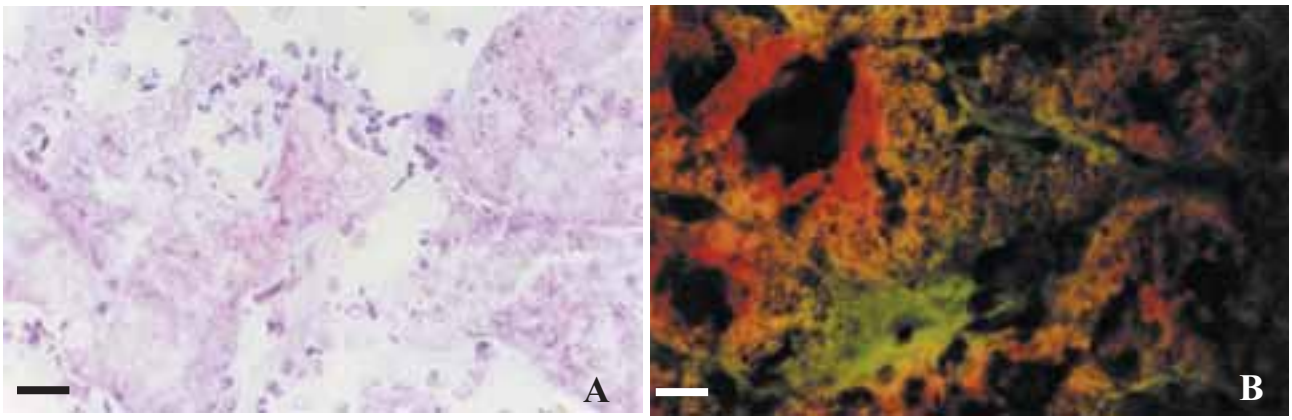

Figure 7. A. Harderian gland section 24 h after laser irradiation. Note the marked cellular fragmentation of the necrotic epithelial cells. There are very few porphyrin-lipid complexes, but edema and presence of granulocytes are observed, HE. **B.** Fluorescence microscopy of a harderian gland section showing that are few remnants of protoporphyrin-lipid-complexes (red fluorescence) 24 h after exposure to laser light. Bar = 15 µm (from Reis *et al.* [43], with permission).

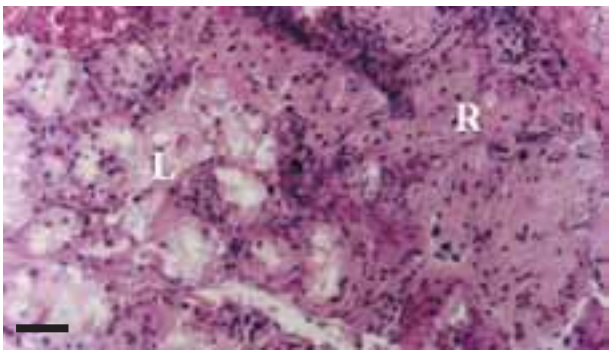

Figure 8. Histological section of a harderian gland treated with non-laser light for 30 minutes (fluency 20.34 J/cm^2). Observe the total acinar destruction on the right **(R)** and the partial destruction on the left **(L)**. This histological section corresponds to the border of the treated zone. Bar = 50 µm (from Mesquita *et al*. [34], with permission).

operated harderian glands (controls) showed neither necrosis nor inflammatory infiltrate. The effects of photodestruction and recovery of the harderian gland in time intervals of 36, 48 and 72 h after HeNe laser irradiation have also been reported by Nicola *et al*. [38]. Figure 8 shows that similar results to those caused by photodestruction of the harderian gland, were also obtained by Mesquita *et al*. [34] using an optical non-laser excitation source.

Conclusions and perspectives

The anatomy and physiology of the harderian gland have been extensively studied in all of the major classes of vertebrates A wide variety of endogenous products is synthesized by this

gland, and several hypotheses have been proposed to describe how the gland functions. However the possibility of using the harderian gland to investigate tissue photodestruction remains poorly studied [34,38,43]. As discussed here the harderian gland may be a useful model for theoretical and experimental studies on photodynamic processes, particularly with regard to the diagnosis and treatment of benign or malignant tissue alterations. Since photodestruction is dependent on light fluence, light wavelength, and oxygen concentration, we can foresee the use of harderian gland for experiments to explain the PDT dependence on these parameters. In the particular case of oxygen concentration dependence, an experiment is under progress, conducted by one of the authors of this paper (EMDN) using the same hyperbaric chamber described by JH Nicola *et al*. [37].

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